EFFECTS AND MECHANISMS OF INTERLEUKIN-10 PROMOTER POLYMORPHISMS ON HIV-1 SUSCEPTIBILITY AND PATHOGENESIS

by

Dshanta Dyanedi Naicker

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Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy in the Discipline of Immunology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal

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PREFACE

The experimental work described in this dissertation was carried out in the HIV Pathogenesis Programme (HassoPlattner Research Laboratory), Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa and The Ragon Institute of MGH, MIT and Harvard, Massachusetts General Hospital-East, 149 13th Street, Charlestown, Massachusetts, from August 2008 to December 2011, under the supervision of Professor Thumbi Ndung'u.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it is duly acknowledged in the text.

Signed: _____ Date: ____ January 21, 2013_____
Dshanta D. Naicker (candidate)

Signed: __  ___ Date: ____January 21, 2013_____
Professor Thumbi Ndung'u (supervisor)

Plagiarism:

DECLARATION

I....Dshanta Dyanedi Naicker.....declare that
(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

(ii) This dissertation has not been submitted for any degree or examination at any other university.

(iii) This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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a) their words have been re-written but the general information attributed to them has been referenced;

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(vi) This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

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My love and soul mate Prishaun, and my family, thank you for always being there for me, your love, encouragement, support and understanding are immensely appreciated...thank you for believing in me.

ABBREVIATIONS

AIDS : Acquired Immune Deficiency Syndrome

CAI : CAPRISA Acute Infection

CFSE : Carboxyfluorescein Succinimidyl Ester

FCS : Fetal Calf Serum

HAB : Human Antibody Serum

HAI : HPP Acute Infection

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV : Human Immunodeficiency Virus

IFN-γ : Interferon-gamma

IL : Interleukin

ml : millilitres

ml : microlitres

ng : nanograms

PBMCs : Peripheral Blood Mononuclear Cells

PBS : Phosphate Buffered Saline

PCR : Polymerase Chain Reaction

pg : picograms

rpm : Revolutions Per Minute

SK : Sinikithemba

SNP : Single Nucleotide Polymorphism

TNF-α : Tumour Necrosis Factor-alpha

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ABSTRACT

HIV infection has risen to pandemic proportions. Interleukin-10 (IL-10), a potent anti-inflammatory cytokine has been shown to enhance the establishment and persistence of chronic viral infections through inactivation of effector antiviral immune responses and it may also directly influence HIV-1 replication in cells of diverse lineages. *IL-10* promoter polymorphisms have been shown to affect HIV-1 susceptibility and pathogenesis. However, the underlying mechanisms are poorly understood. We investigated the relationship between *IL-10* promoter variants, plasma IL-10 levels, and markers of disease outcome in chronically HIV-1-infected individuals. To investigate the mechanistic role of IL-10 and its genetic variants on HIV pathogenesis, we studied markers of activation on B cells, $CD4^+$ and $CD8^+$ T cells, and assessed effects on $CD4^+$ T cell proliferation with and without blockade of the IL-10 pathway.

We used Taqman genotyping assays to genotype three *IL-10* promoter single nucleotide polymorphisms (SNPs) in our study cohort. Baseline plasma IL-10 levels were measured using Luminex technology for 112 individuals. Viral load, $CD4^+$ T cell counts and cytotoxic T lymphocyte (CTL) immune responses were measured at baseline. The rate of $CD4^+$ T cell decrease was calculated in 300 individuals with a median follow-up of 25 months. CD38, CD95, Ki67, IgG and PD-1, markers of activation or exhaustion were measured on B cells, and CD38, CD95, Ki67, HLA-DR and PD-1 were measured on $CD4^+$ and $CD8^+$ T cells in a subset of 63 individuals. $CD4^+$ T cell proliferation was measured using Carboxyfluorescein succinimidyl ester (CFSE) assays, following IL-10 receptor blockade in a subset of 31 individuals.

The *IL-10* -1082G, -592A and -3575 variants were observed at frequencies of 0.3, 0.34 and 0.23 respectively, in our study cohort. Plasma IL-10 levels were significantly higher in the -1082GG group than in the combined AA/AG group (p=0.0006). There was a significant association between the 592AA genotype and a greater breadth of CTL responses compared to the CC and CA (p= 0.002 and 0.004 respectively). The -592AA genotype associated significantly with an attenuated loss of CD4 cells (p= 0.0496), with -592AA having the least change in CD4 cells per year. The median expression of HLA-DR, a marker of T cell activation was significantly higher in the-1082AA group for CD8 cells (p= 0.047), and the -592AA group for CD4 T cells (p= 0.01). The median expression of IgG on the surface of B cells was significantly higher in the -1082GG genotype and the -592CC genotype (p=0.0183 and 0.0659 respectively). Overall, *IL-10* variants correlated with IL-10 expression and CD4 decline during chronic HIV-1 infection. *IL-10* promoter variants may influence the rate of HIV-1 disease progression by regulating IL-10 levels, which in-turn, may affect the breadth of CTL responses. Furthermore, the increased expression of HLA-DR and PD-1 on $CD8^+$ and $CD4^+$ T cells, indicates that lower IL-10 levels are associated with increased immune activation and immune exhaustion. The increased expression of IgG on B cells, suggests that in a setting of lower IL-10, there is possibly a bias towards a Th2 immune response. These data suggest a significant role for *IL-10* genetic variants and IL-10 in HIV pathogenesis. Further studies to determine whether and how the IL-10 pathway may be manipulated for therapeutic or vaccine strategies for HIV are warranted.

ETHICAL APPROVAL

The three study groups included have full ethical approval, from the Ethics Committee of the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal; and informed patient consent was obtained for these studies. Listed below are the study titles as well as the reference numbers.

CAPRISA Acute Infection cohort

Title of study: Viral set point and clinical progression in HIV-1 subtype C infection: the role of immunological and viral factors during acute and early infection.

Reference number: E013/04

HPP Sinikithemba Cohort

Title: Mechanisms of HLA-Associated Control and Lack of Control of HIV Infection

Reference number: E028/99

HPP Acute Infection Cohort

Title: Characterisation of the evolution of adaptive immune responses in acute HIV clade C virus infection

Reference number: E036/06

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Chapter 1

Literature Review

Chapter 1: Literature Review

1.1 INTRODUCTION

We have come along approximately three decades since HIV was first described and the proportion of infected individuals has risen to pandemic proportions. According to the 2009 UNAIDS Global Report: AIDS epidemic update (UNAIDS, 2009), almost 60 million people have become infected with HIV since the beginning of the epidemic, and 25 million deaths have resulted from HIV-related causes. About two-thirds (67%) of the world's HIV infected individuals are from the sub-Saharan African region, which includes South Africa (see Figure 1.1.1). Of the infections among children worldwide, 91% of new infections occur in the sub-Saharan African region. Figure 1.1.2 shows an illustration of the disproportionate global distribution of HIV infection in 2004.

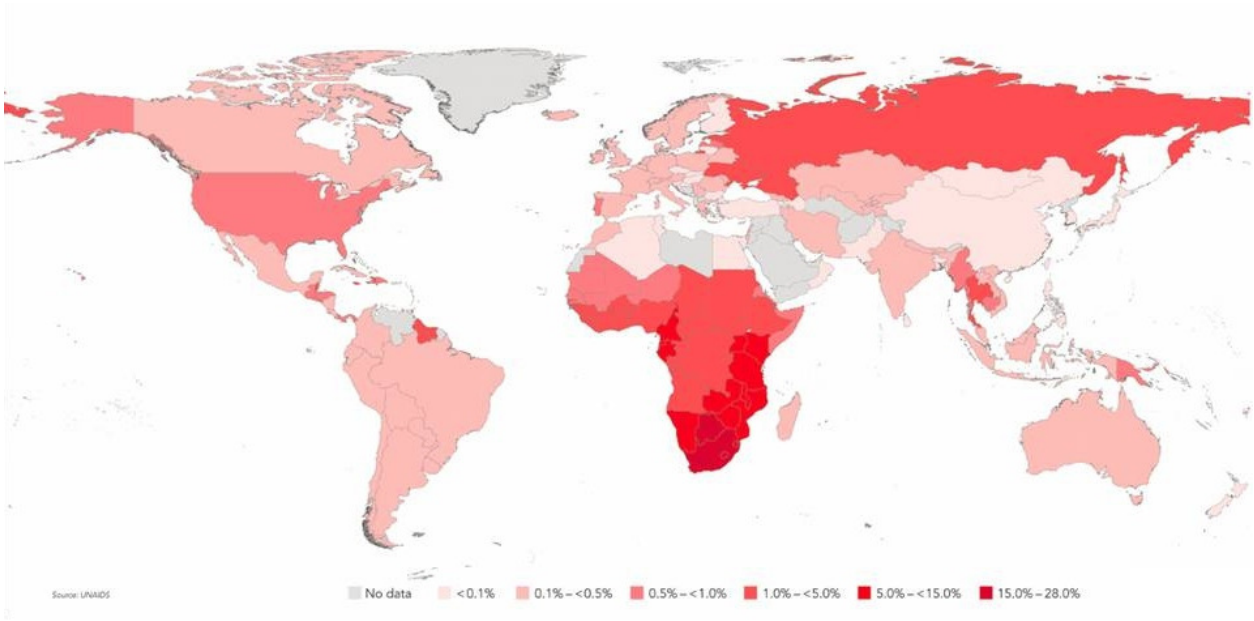

Figure 1.1.1 The global distribution of HIV infection (taken from UNAIDS (UNAIDS, 2010)). The shading of the different geographic regions represents the adult HIV prevalence in 2009. The darker the shading, the larger the number of HIV-infected adults in that region.

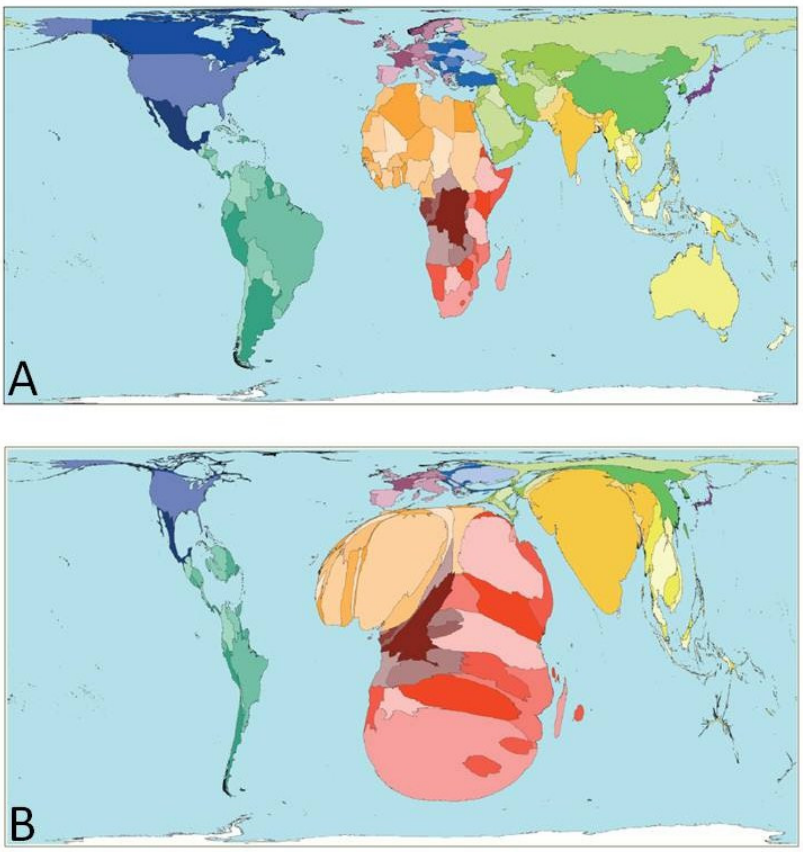

Figure 1.1.2 Global HIV prevalence in 2004 (taken from www.worldmapper.org (Newman, 2006)). Colour is used to group the different territories into 12 geographical regions. The shading is consistent to allow easy visual comparison between the maps. A) This map of the world shows the standard land distribution of the different regions. This is used as a reference map. B) This distorted map of the world is a re-projection of the world, showing each country in proportion to the HIV prevalence in that region in 2004.

Investigation of the underlying biological mechanisms of HIV infection could lead to a preventative vaccine or cure. Studies show that the disproportionate global distribution of HIV infection could be due to different biological factors (Abdool Karim et al., 2007, Cohen et al., 2008, Quinn, 1996). Socio-economic factors such as poor nutrition, legal and economic disadvantages may make populations more vulnerable to HIV infection and spread (UNAIDS,

2009). HIV has different subtypes and strains, that are distributed geographically, which may contribute to the disproportionate distribution of HIV infection. The genetic variation of the population itself may play a role in HIV susceptibility and pathogenesis an example of which are single nucleotide polymorphisms (SNPs). Previous studies have shown that SNPs within the interleukin-10 (IL-10) promoter region of the gene may influence HIV susceptibility and pathogenesis. Understanding the role of IL-10 in a South African population could help with identifying the specific mechanisms involved in facilitating HIV transmission and progression to clinical disease.

1.2 HUMAN IMMUNODEFICIENCY VIRUS

HIV is a lentivirus that targets certain cells of the human immune system. Specifically, HIV attacks helper T-cell lymphocytes, macrophages and dendritic cells (Wallace, 1996). When these vital cells of the immune system are targeted and become depleted, the immune system becomes compromised, leading to co-infections and eventually Acquired Immune Deficiency Syndrome (AIDS).

1.2.1 SUBTYPES & GLOBAL DISTRIBUTION OF HIV

Several factors drive HIV-1 genetic heterogeneity, for example the reverse transcriptase lacks a proofreading ability, the rapid replication of HIV-1 *in vivo*, host immune pressure, and recombination during replication (Buonaguro et al., 2007). Based on the genetic make-up of HIV-1, phylogenetic analyses have led to the classification of HIV-1 into four genetic groups, i.e. M (major), O (outlier) and N (non-M, non-O) (Buonaguro et al., 2007, Hemelaar et al., 2006, McCutchan, 2006, Simon et al., 2006) and the more recently identified P group (Plantier et al., 2009, Vallari et al., 2011). HIV-1 group M is responsible for the majority of infections worldwide. Group M is further divided into subtypes or clades (see Figure 1.2.1), as well as recombinant forms.

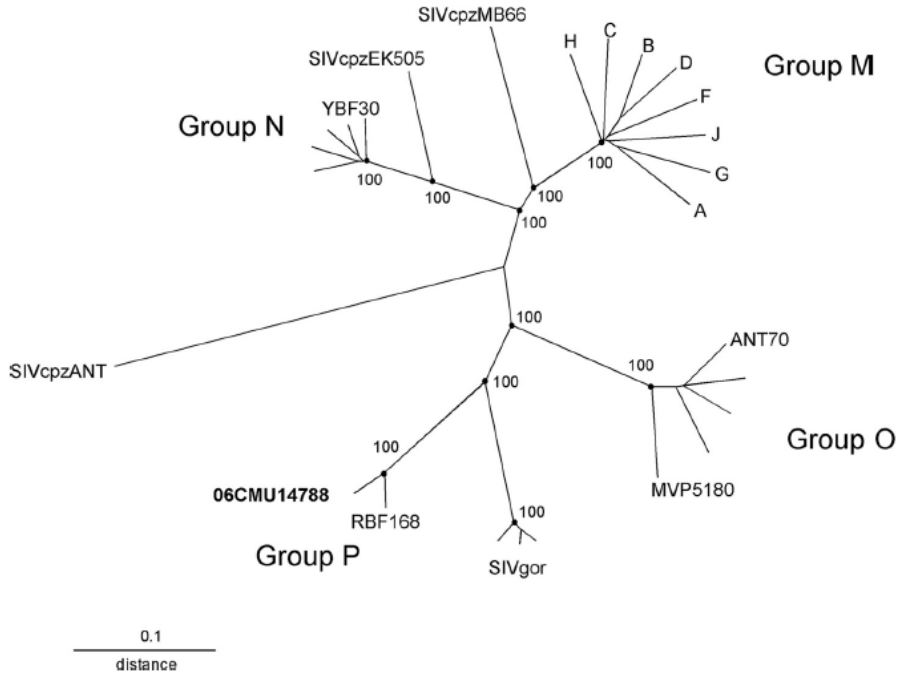

Figure 1.2.1 Phylogenetic tree of HIV-1 groups and subtypes (taken from Plantier (Plantier et al., 2009)). HIV-1 is divided into 4 groups based on genetic analyses, with group M accounting for the majority of infections worldwide. Group M is further divided into subtypes, or clades. Intra-subtype variation can be between 15–20%, while inter-subtype variation is approximately 25–35%, depending on the subtypes and genome regions investigated.

The recombinant forms can be divided into circulating recombinant forms (CRFs) or unique recombinant forms (URFs). With almost all regions of the world being affected by this pandemic, group M dominates infection globally, and group O and N resulting in a small minority of infections (see Figure 1.2.2). There is an unequal geographic distribution of subtypes globally with subtypes A, B and C dominating infection globally (see Figure 1.2.3). Subtype C is responsible for 50-60% of infections worldwide, and is concentrated in Southern and East Africa and India (Buonaguro et al., 2007, Hemelaar et al., 2006, McCutchan, 2006, Simon et al., 2006).

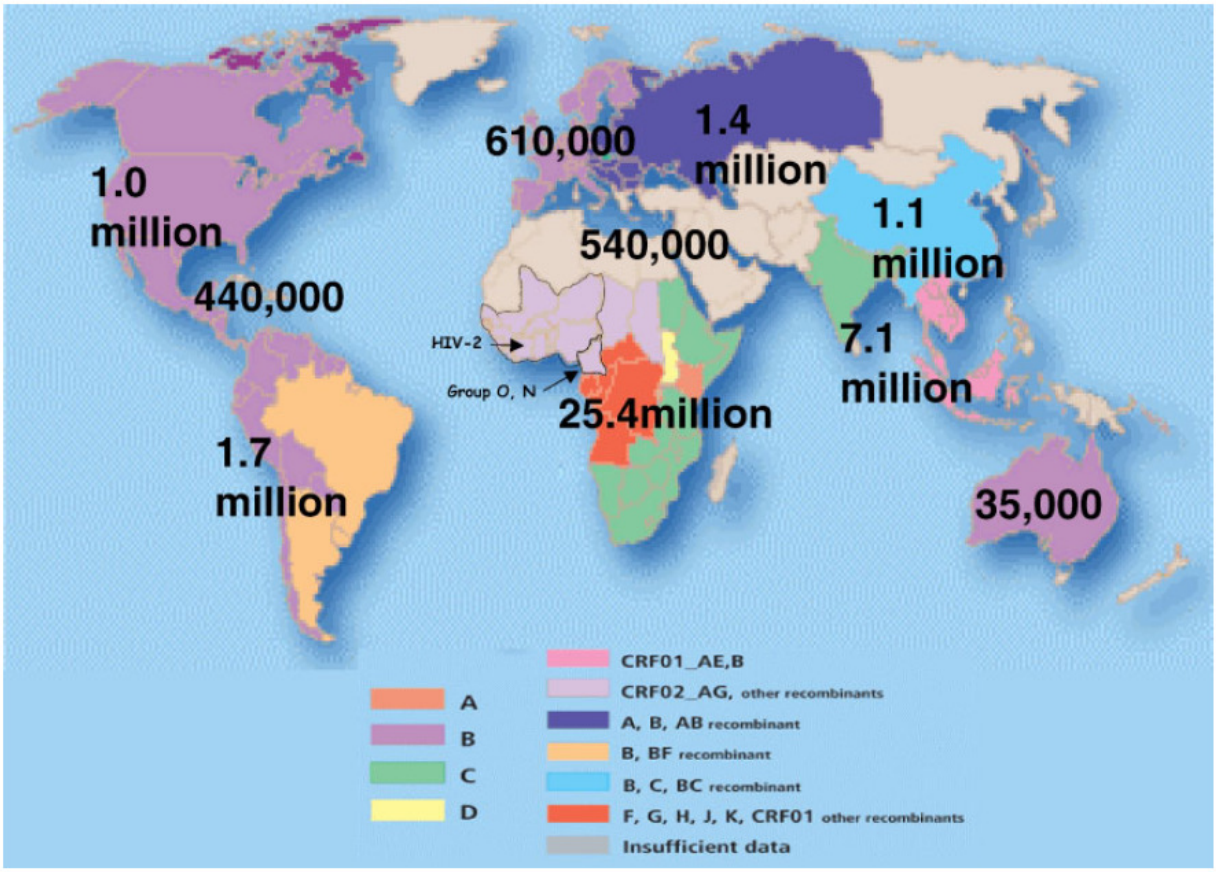

Figure 1.2.2 Geographic distribution of HIV groups, subtypes and recombinant forms globally (taken from (McCutchan, 2006))**.** This map shows the distribution of the subtypes found in different regions globally.

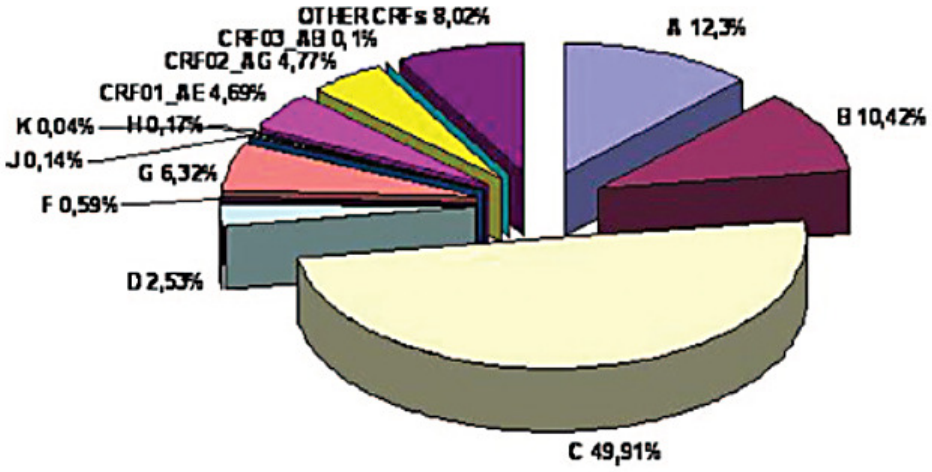

Figure 1.2.3 Global prevalence of HIV-1 subtypes (taken from Buonaguro (Buonaguro et al., 2007)). This is a global distribution of subtypes. Subtypes A, B and C cause the majority of infections worldwide with subtype C being responsible for 50-60% of infections globally.

1.2.2 STRUCTURE

The HIV virus is spherical in shape (see Figure 1.2.4). Free virus particles, known as virions, can be 110nm in diameter (Richman, 2003). HIV contains a protein core or capsid which is surrounded by a lipid bilayer membrane or envelope (Wallace, 1996).

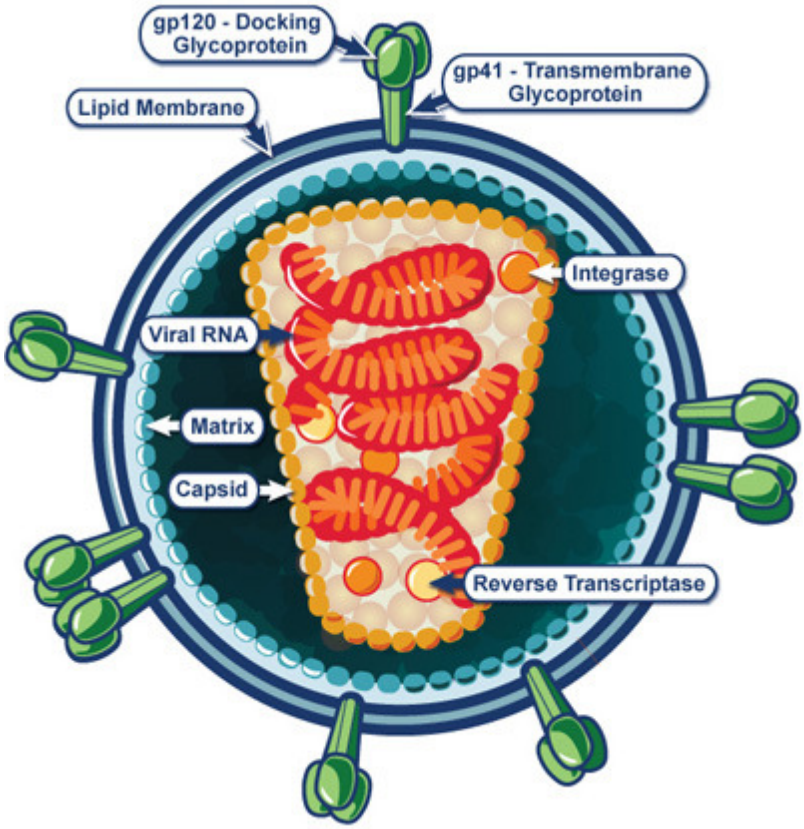

Figure 1.2.4 Structure of HIV (taken from http://www.niaid.nih.gov (NIAID), accessed 30 September 2011). The virus consists of an outer envelope (phospholipid layer) which contains different sized glycoproteins, i.e. either gp120 or gp41. The core of the virus contains the RNA material and accessory enzymes.

Studies suggest that approximately 72 glycoprotein structures are embedded along the lipid bilayer membrane (Richman, 2003). Each glycoprotein trimer is composed of envelope surface glycoprotein gp120, and the transmembrane glycoprotein gp41. The conical shaped protein capsid is found within the envelope. Two copies of the HIV single-stranded RNA genome are found within the capsid, along with several enzymes that are required for host cell infection and viral replication (Richman, 2003). Enzymes required for infection and early viral replication include integrase, reverse transcriptase and protease.

1.2.3 GENOMIC ORGANIZATION

HIV-1 contains 2 copies of single-stranded RNA, each of which are about nine kilobases (kb) long. Each of these strands contains the HIV-1 genome and genetic information encodes all the viral components and proteins (Richman, 2003). During HIV replication, the virus is copied and converted into double-stranded DNA by the process of reverse transcription. Duplication of sequences occurs within the long-terminal repeat (LTR) during reverse transcription, resulting in a slightly longer viral DNA genome of around 10 kb (Richman, 2003). LTRs are essential for the integration into the host cell DNA, and contain gene regulatory protein binding sites which control viral gene expression (Janeway, 2005). The double-stranded viral DNA is referred to as proviral DNA once it is integrated into the host cell by the enzyme integrase. Once integrated, the viral proteins are then expressed during transcription and translation.

The HIV genome is composed of nine genes flanked by LTRs (see Figure 1.2.5). Retrovirus genomes all contain 3 essential genes, i.e. *gag*, *pol*, and *env*. These three genes encode viral structural proteins and are vital for viral replication. The remaining six genes are involved in viral replication and infectivity.

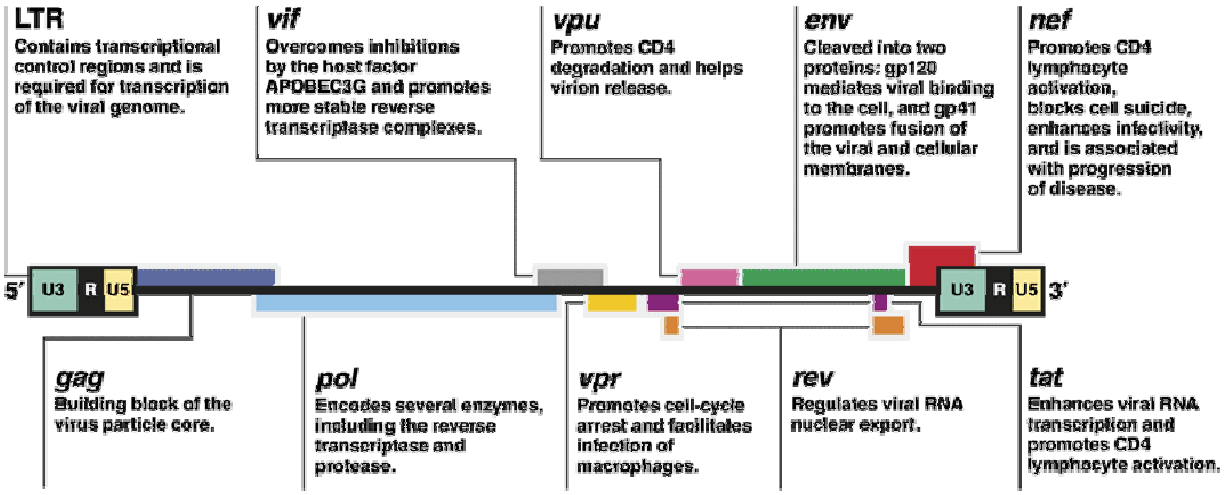

Figure 1.2.5 Genomic structure of HIV (taken from www.gladstone.ucsf.edu (Institutes), accessed 30 September 2011)**.** The HIV genome consists of nine genes flanked by LTRs. Three genes, *gag*, *env* and *pol* are common to all retroviruses, and the remaining six genes have regulatory or accessory functions in viral replication and infection.

The *gag* (group-specific antigen) gene encodes for structural proteins of the viral core, as well as matrix (involved in virion assembly and structure), capsid (forms the conical shaped core) and nucleocapsid (found within the core, and contains reverse transcriptase and integrase) protein domains. The *pol* (polymerase) gene encodes enzymes such as reverse transcriptase, protease and integrase, which are involved in viral replication and integration. The *env* (envelope) gene encodes the viral envelope glycoprotein. The gp160 precursor is cleaved to form the transmembrane glycoprotein gp41 (required for virus fusion and internalisation), and the surface glycoprotein gp120 (binds CD4 and CCR5). The *rev* (regulator of viral expression) gene encodes for proteins involved in processing and expression of viral transcripts. The *tat* (transactivator) gene encodes for proteins that are involved in the positive regulation of transcription, and is essential for viral replication. The *nef* (negative-regulation factor) gene encodes proteins that enhance viral replication and decreases the expression of CD4, and MHC Class I and Class II.

The *vif* (viral infectivity factor) gene encodes for proteins that affect viral particle infectivity and is required for efficient viral replication. The *vpr* (viral protein R) gene encodes for proteins that are involved in transporting the DNA to the nucleus and enhances virion production. Vpr also initiates cell cycle arrest, allowing for efficient viral replication. The *vpu* (viral protein U) gene encodes for proteins that encourage intracellular CD4 degradation and improves virion release. (Janeway, 2005, Richman, 2003)

1.2.4 PATHOGENESIS OF HIV-1

HIV replication occurs within a host cell, as it utilises host enzymes, amino acids, ribosomes and energy to produce components that will be assembled into new virions. The spikes on the HIV-1 envelope are formed by two glycoproteins. The gp120 glycoprotein is the surface glycoprotein and gp41 is the transmembrane protein. As shown in Figure 1.2.6, entry into the cell occurs when the gp120 surface glycoprotein binds to the CD4 receptor on the host cell (Simon et al., 2006, Wallace, 1996). This triggers ensuing interactions between the virus and chemokine receptors such as CCR5 and CXCR4. Fusion of the two membranes occurs within minutes and the viral core is then released into the cytoplasm of the host cell. The virus core then disassembles, and the single-stranded RNA genome is then reverse (*retro*) transcribed into DNA by the viral enzyme reverse transcriptase (Rhoades, 1996). It is during this stage of reverse transcription that viral variants may occur as a result of the lack of proofreading ability of reverse transcriptase (Simon et al., 2006). The viral DNA is then incorporated into the host cell's genome by the viral enzyme integrase. New viral RNA, proteins and particles are generated at the host's expense. The newly produced viral components are then transported to the host cell's plasma membrane, where they are then packaged into new virions which bud from the surface of the cell. This

disrupts normal cell functions and leaves the host cell's plasma membrane perforated, ultimately leading to the death of cells that are essential to the immune response (Wallace, 1996).

$CD4^+$ helper T cells are the main target of HIV. These cells are key participants in cell-mediated and antibody-mediated immune responses. Healthy HIV-negative individuals contain about 1000 $CD4^+$ T cells per cubic millimeter of blood, i.e. 1,200 cells/mm^3 (Janeway, 2005, Rhoades, 1996). Gradual decline in $CD4^+$ T cells results in a compromised immune defense, allowing for the development of opportunistic infections or malignancies.

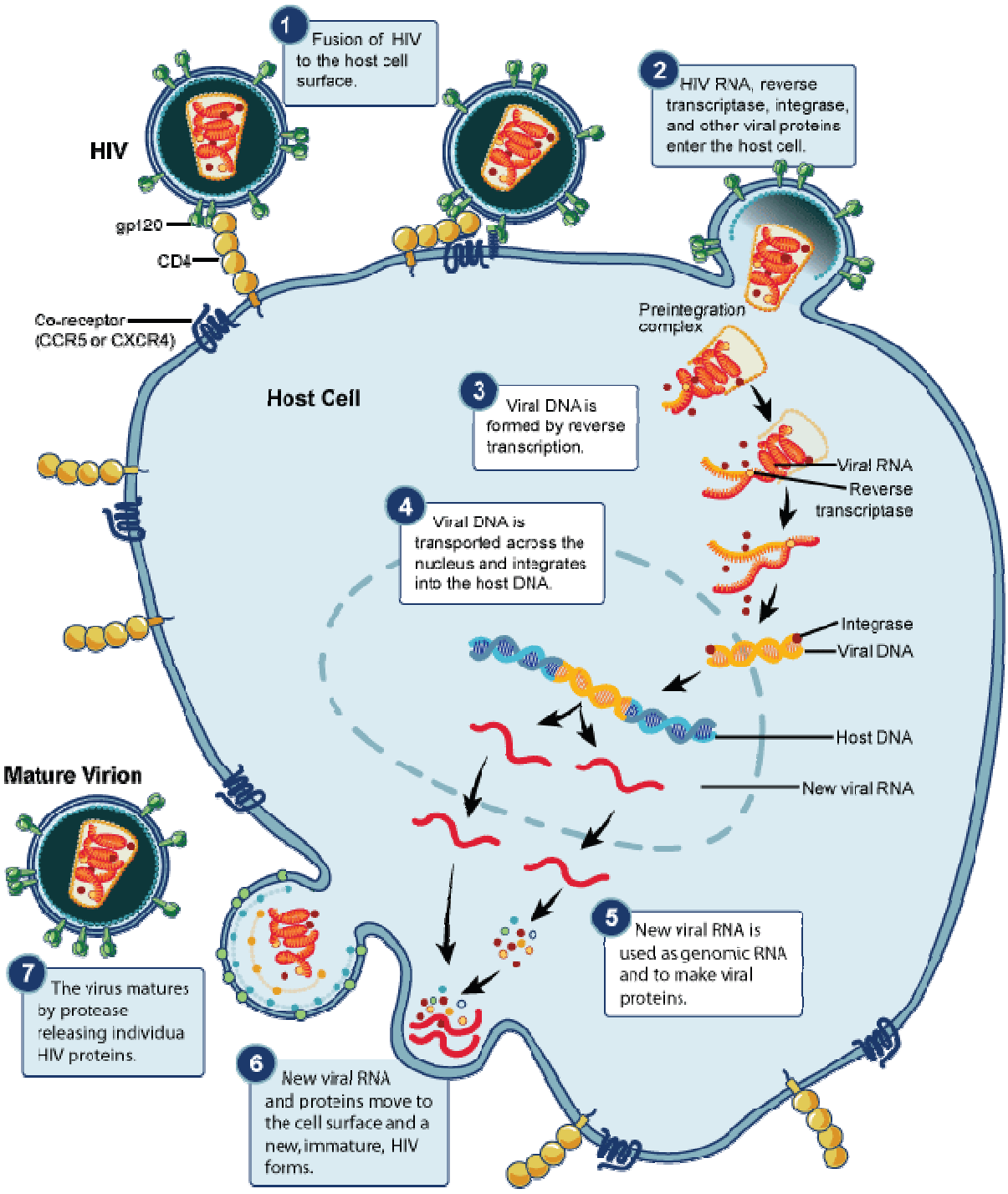

Figure 1.2.6 Life cycle of HIV (taken from http://www.niaid.nih.gov (NIAID) accessed 30 September 2011). HIV enters the cell, where replication occurs. New virions bud from the surface of the cell and continue to infect other cells.

The acute phase of HIV infection is usually the first 2-4 weeks after infection with HIV. During this phase 80% of individuals will experience flu-like symptoms (Janeway, 2005, Roitt, 2000, Snustad, 1999). During this initial phase of HIV infection the population of $CD4^+$ T cells declines, with an increase in plasma levels of HIV (see Figure 1.2.7).

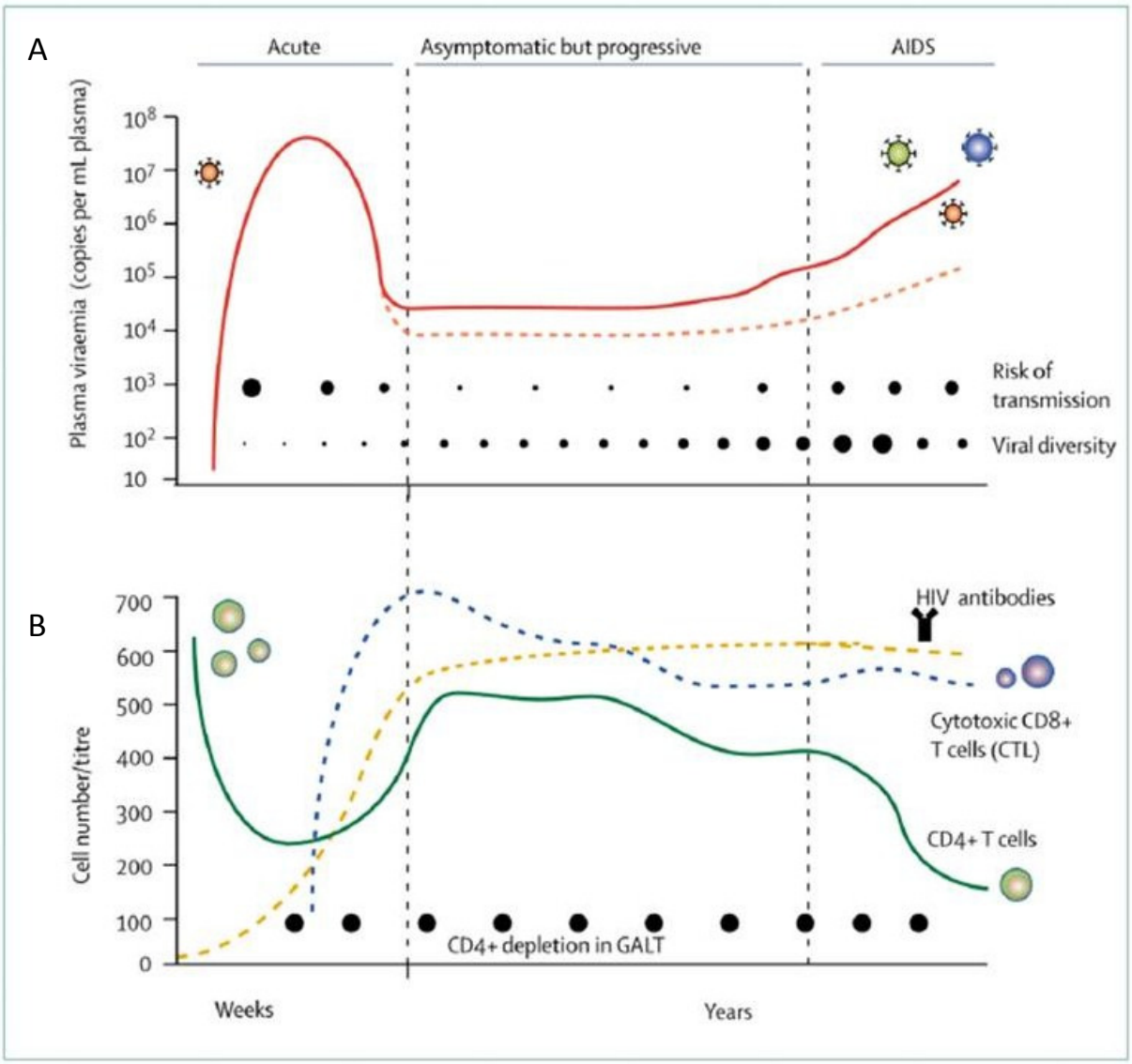

Figure 1.2.7 The course of HIV-1 infection (taken from Simon (Simon et al., 2006)). (A) Acute HIV infection results in a rise in plasma viremia and usually decreases to a viral set point over time. The risk of transmission is higher during the early stages of infection. Viral diversity

increases over time. (B) As plasma viral loads increase (seen in A) $CD4^+$ T cell counts decrease. As the HIV antibodies develop and CTL responses start to appear, when viral loads start to decrease.

Within 2-6 weeks of infection, HIV antibodies to core and surface proteins can be detected using enzyme-linked immunoassays (Roitt, 2000). The cytotoxic T-cell response is crucial in controlling viremia (Koup et al., 1994) . These responses peak then decline after CD4 T cell counts rebound. Following acute infection, the viral load reaches a set point, which is usually a predictor of the rate of disease progression (Lyles et al., 2000, Mellors et al., 1996, O'Brien et al., 1996, Schacker et al., 1998). An asymptomatic phase follows acute infection, where persistent viral replication continues (Bailey et al., 2006, Koup et al., 1994). Despite efforts to fight the infection by the immune system, the population of $CD4^+$ T cells gradually diminishes. It can take between 1-20 years to reach clinical disease when opportunistic infections begin to appear.

In order to become a successful parasite, the final phase in the HIV life cycle is to be passed onto a second host, where it can infect and replicate again. HIV is a sexually transmitted infection, and can be passed on from one individual to the next via semen and vaginal secretions (Wallace, 1996). HIV can also be passed on through blood on hypodermic needles used to inject intravenous drugs. Mother-to-child infection can also occur across the placenta and to infants from breast milk.

The typical course of infection explained here can however vary widely between individuals. Most individuals infected with HIV will follow this typical course of infection and develop AIDS, eventually leading to opportunistic infections and death. However, some individuals,

known as long-term non-progressors (LTNPs) are HIV-positive individuals that have the ability to control the infection without antiretroviral therapy. The mechanisms responsible for LTNP are poorly understood but may involve multiple immune mechanisms such as humoral immunity, for example LTNPs may develop antibodies against HIV proteins, which may help in controlling the virus to relatively low levels of viremia (Braibant et al., 2006, Cao et al., 1995, Carotenuto et al., 1998, Montefiori et al., 1996, Pereyra et al., 2009, Pilgrim et al., 1997), also host genetics may play a role in controlling HIV pathogenesis, for example protective Human Leukocyte Antigen alleles may assist in controlling HIV pathogenesis. It has also been established that certain individuals remain seronegative despite being highly exposed to HIV (Beyrer et al., 1999, Kulkarni et al., 2003, Rowland-Jones et al., 1999). The ability to control or be resistant to HIV infection may lie in the host genetic factors of the individual.

1.2.5 HOST GENETIC FACTORS

It has been shown that individuals exposed to HIV have a differential propensity to become infected, and once infected the rate of progression to severe immunodeficiency leading to AIDS and eventually death differs between individuals. Allelic variants in the human genome may play a role in the regulation of HIV susceptibility or resistance to infection, and the pathogenesis of HIV progression. Several genes have been identified to influence the outcome of HIV-1 exposure or infection, and O'Brien and Nelson termed these as **A**IDS **r**estriction **g**enes (ARGs) (O'Brien and Nelson, 2004). Table 1.2.1 shows the ARGs and their effects, as well as a comparison in allelic frequencies between African Americans and European Americans (Winkler et al., 2004). These host restriction factors vary in the mode of inheritance, i.e. dominant,

recessive or codominant; the phase of HIV infection in which they play a role and the stage that the effect is evident (O'Brien and Nelson, 2004).

Two cytokine genes have been identified as ARGs, i.e. interleukin-10 (IL-10) and interferon-γ (IFN-γ) (O'Brien and Nelson, 2004, Winkler et al., 2004). Polymorphisms in these cytokine genes have been shown to play a role in HIV susceptibility and HIV-1 pathogenesis. *IL-10* promoter polymorphisms have been shown to limit HIV-1 infection and affect the rate of disease progression, while *IFN-γ* allelic variants affect the rate of disease progression. Polymorphisms in these genes that restrict HIV infection are mainly due to single nucleotide polymorphisms (SNPs). SNPs are single base mutations in DNA.

Table 1.2.1 Comparison of the effects of ARGs on African Americans and European Americans (taken from (Winkler et al., 2004))

	Allele or factor	Gene function	Mutation or variant allele effect	Effect	Relative risk or hazard[a]	
					AA	EA
Protective factors						
CCR5	Δ32	HIV-1 coreceptor	Limits HIV-1 cell entry	HIV-1 infection resistance	NA	0.03
				Delays AIDS	NA	0.38
CCR2	64I	HIV-1 coreceptor	Blocks dimerization of receptor with CXCR4?	Delays AIDS	1.12	0.69
SDF1	3′A	CXCR4 ligand	Unknown	Delays AIDS	NA	0.36
CXCR6	E3K	CC receptor	Codon change may alter ligand binding efficiency	Increased survival time after PCP diagnosis	0.37	NA
CCRL2	167Y	CC receptor	Codon change may alter ligand binding efficiency	Resistance to PCP in EA	NE	0.32
MCP1.MCP3. Eotaxin	Hap 7	Immune modifiers	Unknown	HIV-1 Infection resistance	0.6	0.6
HLA	B*27	Ag presentation	Hinders HIV immune escape	Delays AIDS	NA	0.70
HLA	B*57	Ag presentation	Hinders HIV immune escape	Delays AIDS	0.31	0.45
KIR-HLA	KIR3DS1 + HLA Bw4-80I	Receptor and ligand for innate immunity	May aid NK cell action against HIV infected cell	Delays AIDS	0.25	0.59
Susceptible factors						
IL10	5′A	Th2 cytokine	Down-regulates IL10	Accelerates AIDS	NR	1.44
				Promotes HIV-1 infection	NR	1.75
IFNG	179T	Th1 cytokine	Aberrant IFNG regulation	Accelerates CD4 loss, AIDS	2.47	NA
RANTES	In1.1C	CCR5 ligand	Down-regulates RANTES	Accelerates AIDS	1.93	NE
	Haplotype R3	CCR5 ligand		Accelerates AIDS	1.70	2.27
HLA	B*35-Cw*04	Antigen presentation		Accelerates AIDS in EA	NE	2.28
HLA	B35*Px	Antigen presentation	Weak epitope binding may help HIV immune escape	Accelerates AIDS	2.13	2.69
CCR5	+.P1.+	HIV-1 coreceptor	Upregulates CCR5 expression	Accelerates AIDS	2.31 (Dom)	1.79 (Rec
HLA class I	1 locus homozygosity	Antigen presentation	Reduced epitope recognition repertoire	Accelerates AIDS	2.7	1.39
HLA class I	2 or 3 loci homozygosity	Antigen presentation	Reduced epitope recognition repertoire	Accelerates AIDS	NA	4.33
KIR3DS1	KIR3DS1 in absence of ligand	Activating receptor for innate immunity	Poor regulation of NK cell activity	Accelerates AIDS	2.23	1.21

[a]Only one representative RH from three AIDS endpoints (CD4 200, AIDS 1993 or AIDS 1987) assessed is listed.
NA, effective genotype is absent or very rare to assess. NE, no effect; NR, not reported; EA, European Americans; AA, African Americans.

1.3 HUMAN IMMUNE SYSTEM

The human immune system is a multifaceted organisation of cells, tissues and organs that aim to protect the body from potential harmful molecules. Microbes such as bacteria, viruses, parasites and fungi are thought to be the primary source of foreign attacks against the body. The immune system also protects against unusual or defective self-processes such as tumours. Two types of immunity come into play during the immune response, i.e. the innate immune response and the adaptive immune response (see Figure 1.3.1).

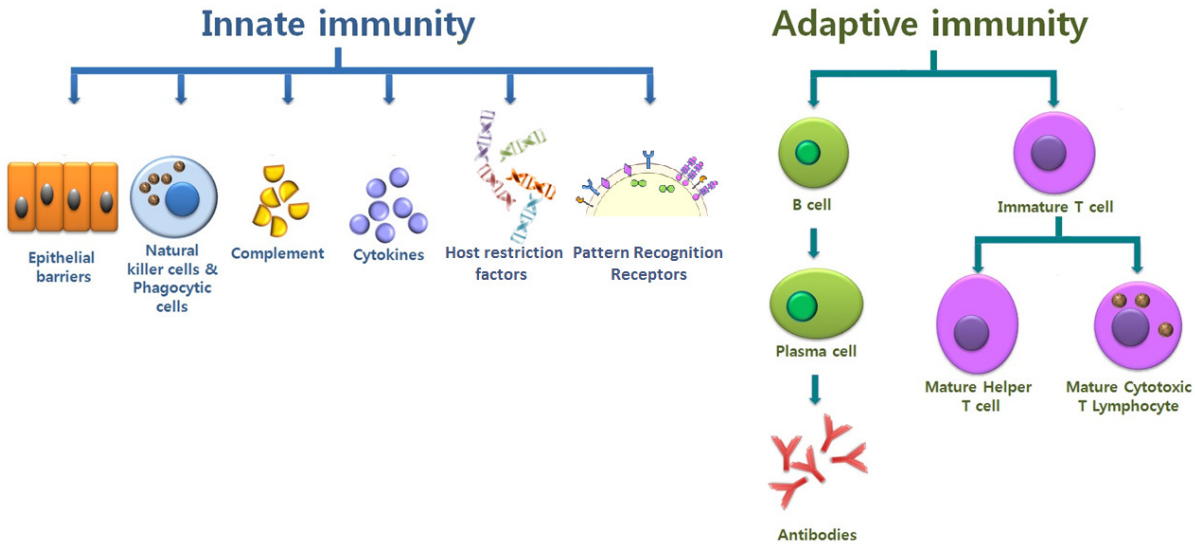

Figure 1.3.1 The Human Immune System. The immune system is divided into two arms, the innate immunity and adaptive immunity. The innate immunity reacts over a short period of time, following pathogen invasion. The innate response involves epithelial barriers, natural killer cells and phagocytic cells, complement, cytokines, host restriction factors and pattern recognition receptors. The adaptive response reacts which largely involves B cells and T cells confers life-long (or long-lived) immunity.

Innate immune responses produce the first line of defense against foreign pathogens (Janeway, 2005, Sherwood, 2001). This arm of the immune response is a combination of nonspecific responses, including cytokine secretion, natural killer (NK) cells and the complement system. Cytokines are chemical messengers of the immune system that relay signals from one cell to another in order to initiate an immune response. NK cells have the ability to non-specifically lyse and destroy virus-infected cells. The complement system, which consists of serum and membrane proteins, is the major effector of the humoral branch of adaptive immunity. The complement system involves a number of proteins that come together to 'complement' antibodies in destroying bacteria. Upon activation by a trigger, proteases cleave specific proteins which release cytokines, this in turn, initiates an amplifying cascade of further cleavages (Delves and Roitt, 2000b, Delves and Roitt, 2000a). Genes aid in natural immunity, as we inherit it. Certain genes, or mutations in genes, enhance the immune response against immune invasion. In HIV infection, host restriction factors play a role in HIV susceptibility and pathogenesis. Another component of the innate immune system is pattern-recognition receptors (PRRs). These receptors are germline-encoded, and are involved in identifying components of foreign pathogens. The innate immune response is the key to initiating the adaptive immune response.

The adaptive immune response is more specific as it can distinguish between millions of different foreign molecules. The adaptive immune response is divided into two branches of immunity: antibody-mediated (also commonly referred to as humoral) and cell-mediated.Lymphocytes are the key cells involved in the adaptive immune response. B cells and T cells are the main types of lymphocytes. Plasma cells are derived from B cells and they are responsible for the antibody-mediated immunity (Rhoades, 1996, Sherwood, 2001,

Snustad, 1999). Plasma cells produce antibodies. Coordination of the antibody-mediated response involves the coordination of $CD4^+$ T cells, antigen-presenting cells such as macrophages, and B cells. This coordination of cells involves cytokines. T cells are involved in the cell-mediated immunity. Cytokines such as interleukins and interferons coordinate the interaction between antigen-presenting cells, such as macrophages and B cells, and $CD4^+$ and $CD8^+$ T cells (Rhoades, 1996).

1.3.1 IMMUNE RESPONSES TO HIV INFECTION

Although the genome size of HIV-1 may seem modest in size being less than 10kb, it has the ability to utilise cellular pathways while neutralising and evading components of the immune system.

During the asymptomatic phase of HIV infection, the viral load is controlled to a quasi-stable level, this could be due to cytotoxic T cells invading and killing productively infected cells found in sites of HIV replication (Janeway, 2005). Another explanation could be that the virus runs out of target cells, i.e. $CD4^+$ T cells, most of which are destroyed and reside in the gut mucosa (Brenchley et al., 2006). It has been shown that high levels of HIV-specific $CD8^+$ T cells correlated with slower disease progression. $CD4^+$ T cells have also been shown to play an essential role in the host's response to HIV infection (Michael, 1999). Evidence shows that a correlation was found between the strength of $CD4^+$ T cell proliferative responses and viral load, strong $CD4^+$ T cell proliferative responses were seen in individuals that did not progress to AIDS (Michael, 1999). $CD4^+$ T cell responses play a central role in controlling infection,

but on the other hand are the main target for HIV, the depletion of these vital cells may explain the inability of the immune system to control the infection.

Evidence that certain individuals may be more resistant to HIV infection and some may respond differently during the course of HIV infection suggests that innate immunity and the natural response may play a role in the natural mechanisms that control HIV resistance and disease pathogenesis. In the last decade in the field of HIV, a significant discovery has been the identification of host restriction factors (also called intrinsic immunity) that play a key role in antiretroviral defense by diverse mechanisms. Host restriction factors like APOBEC3G, Trim5 and tetherin are some of the host restriction factors that have been shown to play a role in HIV-1 infection.

Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) is a cytidine deaminase, which deaminates deoxycytodine to deoxyuridine in viral DNA during reverse transcription, therefore altering the HIV RNA. This results in hypermutation in the plus strand DNA. APOBEC3G has been shown to be a potent inhibitor of HIV replication (Browne et al., 2009, Goila-Gaur and Strebel, 2008, Kao et al., 2007, Xu et al., 2007). However HIV has an accessory protein, Vif, which counteracts the effect of APOBEC3G. Trim5α is another host restriction factor, which targets HIV (Sayah et al., 2004, Stremlau et al., 2004). The process by which Trim5α restricts HIV replication is by recognition of the retroviral capsid and promoting the premature capsid disassembly (Nisole et al., 2005, Stremlau et al., 2006) . However, human Trim5α has limited efficacy and is not as potent as primate Trim5α. Tetherin, also referred to as BST-2, is a host restriction factor which restricts HIV virion release from infected cells by tethering nascent enveloped virions to the cell

membrane. However, the HIV accessory protein Vpu down-regulates tetherin on the cell surface . (Hammonds et al., 2012, Van Damme et al., 2008)

1.4 CYTOKINES

Cytokines are small secreted signaling proteins usually with molecular weights of between 8-70 kD. These proteins are produced by a variety of cell types and are essential in mediating and regulating both the innate and adaptive immune responses, and inflammation.

The cytokine network is a highly regulated system of cytokines that mediate intercellular communication, and the development and function of the immune response (see Figure 1.4.1). These intercellular communicators are synthesised, released and recognised by immune system cells (Kiyono, 1990). Cytokines mediate the intensity and duration of the immune response, as well as many significant interactions between cells of the immune system (Stites, 1994). These signaling proteins bind to their respective receptors on the cell surface, in response to a stimulus, which then results in the activation of successive signal transduction pathways within a cell (Smith and Humphries, 2009). The binding of the cytokine to its respective receptor initiates specific cytokine action.

Interleukins (IL) are cytokines which are released from T helper (Th) cells of the immune system. There are different subsets of Th cells which produce different sets of cytokines, i.e. Th1 and Th2, and the recently described Th17 subset. Th1 cells produce cytokines such as interferon-gamma (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α) whereas Th2 cells produce cytokines such as IL-4, IL-5 and IL-10. The balance between the secretion of Th1 and Th2 cells may influence the phenotype and outcome of several diseases (Tsiligianni et al., 2005). The Th17 subset of T cells is responsible for producing the cytokine

IL-17, and has been identified to be involved in various autoimmune diseases (Stockinger and Veldhoen, 2007).

Cytokine gene expression is highly regulated. Cytokine expression may vary due to environmental factors, or genetic polymorphisms. Altered cytokine expression has been implicated in various chronic diseases, resistance/susceptibility to infection and pathogenesis and response to treatment of certain diseases (Smith and Humphries, 2009).

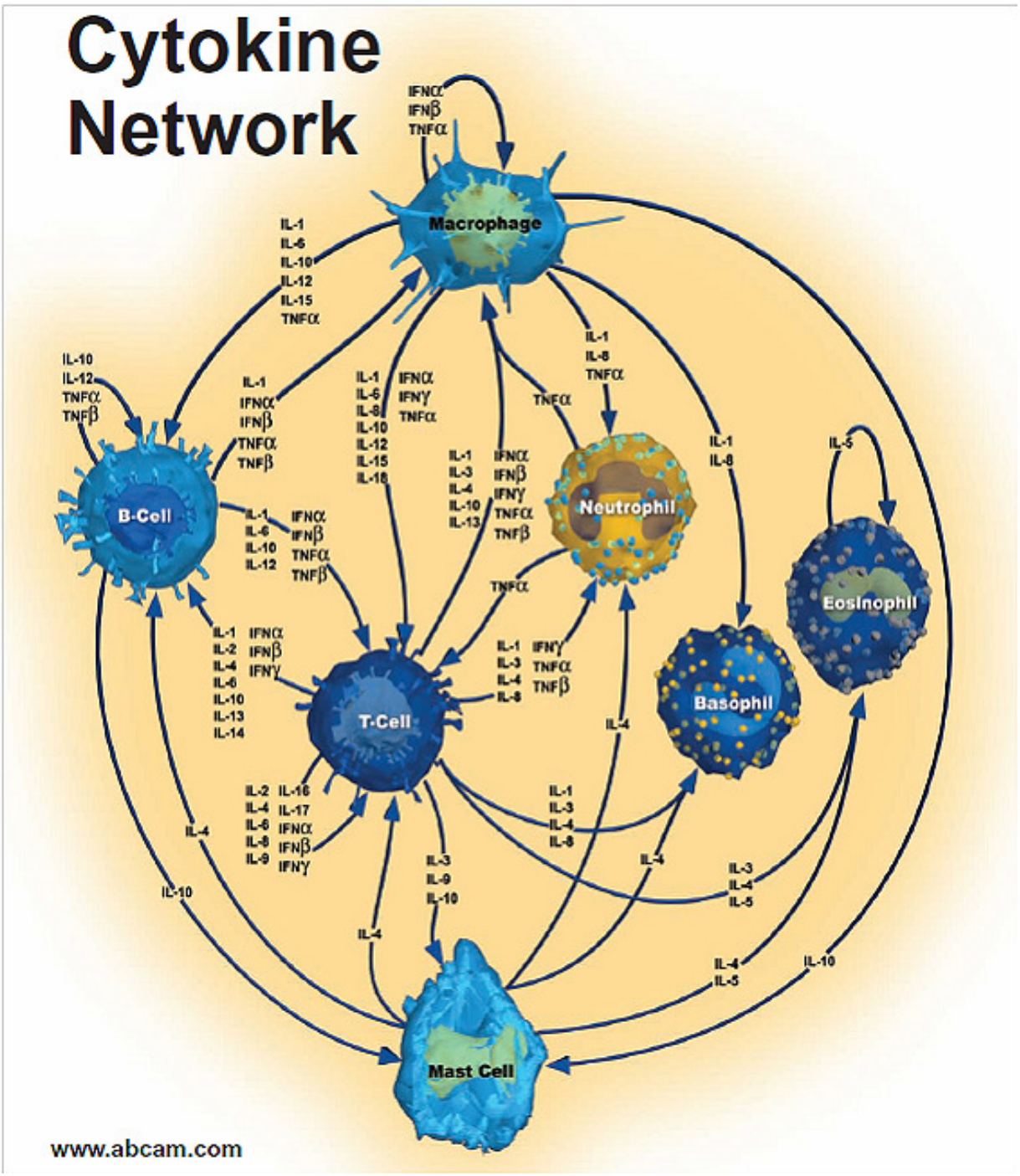

Figure 1.4.1 The cytokine network (taken from www.abcam.com, accessed on 2 October 2011). The cytokine network is an intricate system of a variety of cells and cytokines. Cytokines are intercellular communicators that signal different pathways within different cells.

1.4.1 IL-10

Interleukins are a group of cytokines which are involved in stimulating immune cell proliferation and differentiation. The *IL-10* gene is located on human chromosome 1, between 1q31 and 1q32 (see Figure 1.4.2) (Lazarus et al., 2002). The gene consists of 5 exons, and spans about 5kb. IL-10 prevents unnecessary inflammation during the immune response (Abbas, 2007, O'Farrell et al., 1998). IL-10 is a powerful cytokine that is produced by Th2 cells, monocytes, macrophages, regulatory T cells, and certain activated B and T cells, while the IL-10 receptor is expressed on a range of cells, in particular the immune cells (Asadullah et al., 2003) It has been shown that IL-10 downregulates the expression of major histocompatibility complex (MHC) Class I and Class II antigens, as well as the expression of numerous Th1 cytokines such as IL-1, IL-2, IL-6, IL-8, IL-12 and TNF-α (Ness et al., 2004, Tedgui and Mallat, 2006).

IL-10 has been shown to enhance B cell proliferation, survival and antibody production (Croxford et al., 1998, Hunt et al., 2000). IL-10 is a negative feedback regulator for macrophage activation. Many studies have shown that IL-10 production may vary widely between individuals. Polymorphisms found within the promoter region of the IL-10 gene, which is upstream from the transcription start site, have been found to be associated with differential IL-10 production. These polymorphisms are SNPs and are named based on their position which is upstream from the transcription start site. Studies have focused on three polymorphisms found in the proximal region of the promoter (the region closer to the transcription start site) and three polymorphisms found in the distal region of the promoter (the region further away from the transcription start site). The three proximal *IL-10* polymorphisms

are the -1082 polymorphism (rs1800896), which is an A to G transition; -819 (rs1800871), which is a C to T transition; and the -592 polymorphism (rs1800872), which is a C to A transversion. The -819 mutation T is in complete linkage disequilibrium with the -592 mutation A, therefore these mutations are almost always inherited together, so if there is a mutation at the one position, there will always be a mutation at the other (Stanford et al., 2005). The three distal *IL-10* polymorphisms are -2763 (rs6693899), which is C to A transversion; -2849 (rs6703630), which is a G to A transition; and -3575 (rs1800890), which is a T to A transversion. Previous studies have shown that these polymorphisms can either up-regulate or down-regulate the expression of IL-10. The mutation from A to G at position -1082 has been associated with higher IL-10 production *in vitro* (Turner et al., 1997). Previous reports demonstrate that the wild-type -1082A allele is associated with low IL-10 production, and the mutation -592A allele is associated with low IL-10 production (Hutchinson et al., 1998, Turner et al., 1997). Previous studies have also demonstrated that IL-10 production varies between different IL-10 haplotypes based on positions -1082, -819 and -592 (Eskdale et al., 1998). The GCC/GCC haplotype is associated with high IL-10 production; the GCC/ATA or GCC/ACC haplotypes are associated with intermediate IL-10 production; the ATA/ATA, ATA/ACC or ACC/ACC haplotypes are associated with low IL-10 production (Edwards-Smith et al., 1999).

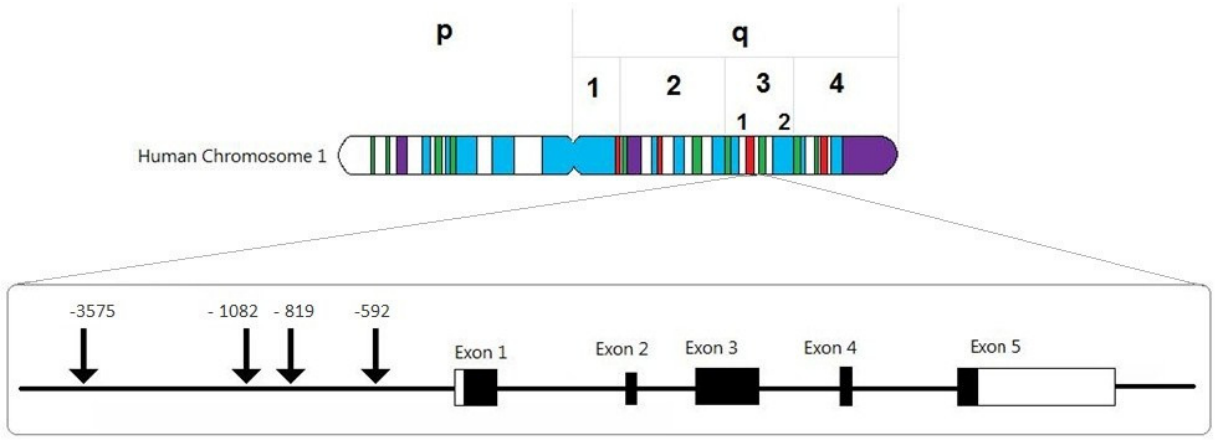

Figure 1.3 Location of Interleukin-10 (*IL-10*) gene. The IL-10 gene is located on the q arm of human chromosome 1, between the region 1q31 and 1q32. The *IL-10* gene spans about 5 kilobases, and consists of 5 exons. The polymorphic region is found within the promoter region, which is upstream from the transcription start site.

1.4.2 IL-10 AND HIV

IL-10 is a pleiotropic cytokine that has various anti-inflammatory and immunosuppressive activities. This potent cytokine has been associated with HIV susceptibility and has been shown to play a role in disease progression to AIDS. However, the underlying mechanisms on how the cytokine may control HIV infection and pathogenesis are not well understood.

As this Th2 cytokine has the ability to down-regulate the expression of Th1 cytokines it has been suggested that it may have adverse effects on HIV infected individuals. The down-regulation of Th1 cytokines may result in the inhibition of robust T cell-mediated responses and the presentation of antigen on the cell surface (Ji et al., 2005). SNPs that affect the

expression of IL-10 production have been shown to play a role in HIV susceptibility and pathogenesis.

IL-10 has been shown to inhibit HIV replication within macrophages and monocytes (Porcheray et al., 2006, Wang and Rice, 2006). Infection within monocytes and macrophages is less productive than it is within $CD4^+$ T cells, as these are the primary target population, however HIV replication within macrophages and monocytes may have significant consequences for HIV transmission and pathogenesis, especially during the late stages of infection, when $CD4^+$ T cells have been depleted (Orenstein et al., 1997).

Previous genetic studies focused on *IL-10* promoter polymorphisms have shown that these SNPs play a role in HIV susceptibility and pathogenesis of infection. In a study by Shin et al in 2000 results showed that *IL-10* promoter polymorphisms affect HIV-1 infection and pathogenesis in a cohort comparing individuals of different ethnicity (Shin et al., 2000). In this study they found a significant association between the -592A polymorphism and an acceleration to AIDS in Caucasians. They also found that low-IL-10-producing genotypes were associated with susceptibility to HIV infection and an AIDS-accelerating effect. In another genetic study of a Zimbabwean cohort of HIV infection (Erikstrup et al., 2007) it was shown that survival was doubled in individuals carrying the -1082G polymorphism and this allele was also associated with an attenuated loss of $CD4^+$ T cells. More recently Oleksyk et al (Oleksyk et al., 2009), showed that European individuals carrying the low-IL-10-producing ATA haplotype had a faster progression to AIDS, however, this was not the case among African Americans. In our previous study on the role of *IL-10* promoter variants in acute HIV infection, we found that individuals with high-IL-10-producing genotypes were less likely to

become infected, but once infected showed a significantly higher median viral load during the first 3 months, than the other *IL-10* variants (Naicker et al., 2009). However this association seemed to reverse as infection progressed.

Mechanistic studies examining lymphocytic choriomeningitis virus (LCMV) in mice showed that IL-10 gene knock-out or signaling blockade enhanced T-cell immune responses, which resulted in rapid viral elimination and the development of antiviral memory T cell responses (Brooks et al., 2006, Ejrnaes et al., 2006). A recent study by Brockman et al, showed that IL-10 receptor blockade *in vitro* within peripheral blood mononuclear cells (PBMCs) from HIV-infected individuals resulted in restoration of proliferative and effector $CD4^+$ T cell function (Brockman et al., 2009). IL-10 is also reported to enhance detrimental deletion of dendritic cells by NK cells, therefore intensifying immune dysfunction in chronic HIV-1 infection (Alter et al., 2010). Taken together, these studies suggest a complex but significant role for IL-10 in HIV pathogenesis. It has been suggested that the IL-10 pathway be manipulated to boost antiviral immune responses and improve vaccine effectiveness (Brooks et al., 2008), therefore there is a clear and urgent need to better understand the underlying mechanisms of IL-10 in HIV pathogenesis, particularly in geographical regions most severely affected by the HIV-1 epidemic, as this may have implications for immunotherapeutic strategies and vaccine design.

1.5 PROJECT AIMS AND STRATEGY

Previous genetic studies have identified IL-10 to be a host genetic factor which restricts HIV susceptibility and pathogenesis. However, these studies have mostly focused on populations in geographic groups where the burden of HIV infection is low. It is important to study the effects of these polymorphisms in a South African setting, to determine the effects of these polymorphisms in this setting of high HIV disease burden. The effects of these mechanisms may vary based on the strain and subtype present, and given that the subtypes found within sub-Saharan Africa are different compared to subtypes found in previous studies done on American and European individuals. Also, the effect of IL-10 may vary depending on the stage of HIV-1 infection. Environments with poor resources and health care are more at risk for infectious diseases to persist at higher rates. It is evident from the global distribution of HIV and co-infections that the incidence of co-infections varies between the West and low income, developing settings such as South Africa (Corbett et al., 2002). Co-infections such as malaria, STIs and tuberculosis have played a major role in enhancing the spread of HIV-1 (Girardi et al., 2000, Samba, 2001, Wasserheit, 1992). Therefore our overarching hypothesis was that IL-10 single nucleotide polymorphisms associated with high IL-10 production are associated with reduced susceptibility to HIV-1 subtype C infection, higher viral loads during the acute or early HIV-1 infection phase and delayed rate of disease progression or loss of $CD4^+$ T cell counts during the chronic/late phase of infection.

The first aim of this study was to determine the allele frequency of three IL-10 SNPs in three study cohorts. The SNPs that were of interest were found at positions -592, -1082, and -3575. The cohorts that were included in this study included individuals at varying stages of HIV

infection, as well as some individuals that were at high risk of infection, but remained HIV-negative. The cohorts that were included in the study were the CAPRISA Acute Infection cohort, the HPP Acute Infection cohort and the HPP Sinikithemba cohort of chronic HIV infection.

The second aim of this study was to investigate the association between *IL-10* promoter polymorphisms and IL-10 levels, as well as the association between *IL-10* variants and the expression of other pro- and anti-inflammatory cytokines *in vivo* during chronic HIV-1 infection. This would give us an indication of cytokine profiles in individuals with different genotypes in an HIV setting. These cytokine profiles were measured in individuals during the chronic phase of infection.

The third aim of this study was to determine the association between *IL-10* variants and different biomarkers of HIV infection. Here we wanted to determine the association between *IL-10* variants and viral load, $CD4^+$ T cell count and $CD4^+$ T cell decline. We also wanted to determine the association between *IL-10* variants and the breadth and magnitude of HIV-1 specific $CD8^+$ T cell immune responses. These were measured at routine visits in individuals at both the acute and chronic phase of infection.

The last aim of this study was to look at the mechanistic effects of these *IL-10* variants and the role they play in an HIV setting. This would help us determine if *IL-10* promoter variants provide either detrimental or beneficial effects. Activation of three different cell populations was measured in individuals in the chronic phase of HIV infection. We determined the association between $CD4^+$ T cell, $CD8^+$ T cell and B cell activation with *IL-10* promoter

variants. We next looked at the association between *IL-10* promoter variants and proliferation. We used IL-10 receptor blockade assays, either with or without stimulation to determine if *IL-10* genotype associated with IL-10 blockade. Figure 1.5.1 below shows an overview of the study strategy. The following chapters will present the different aims and objectives separately.

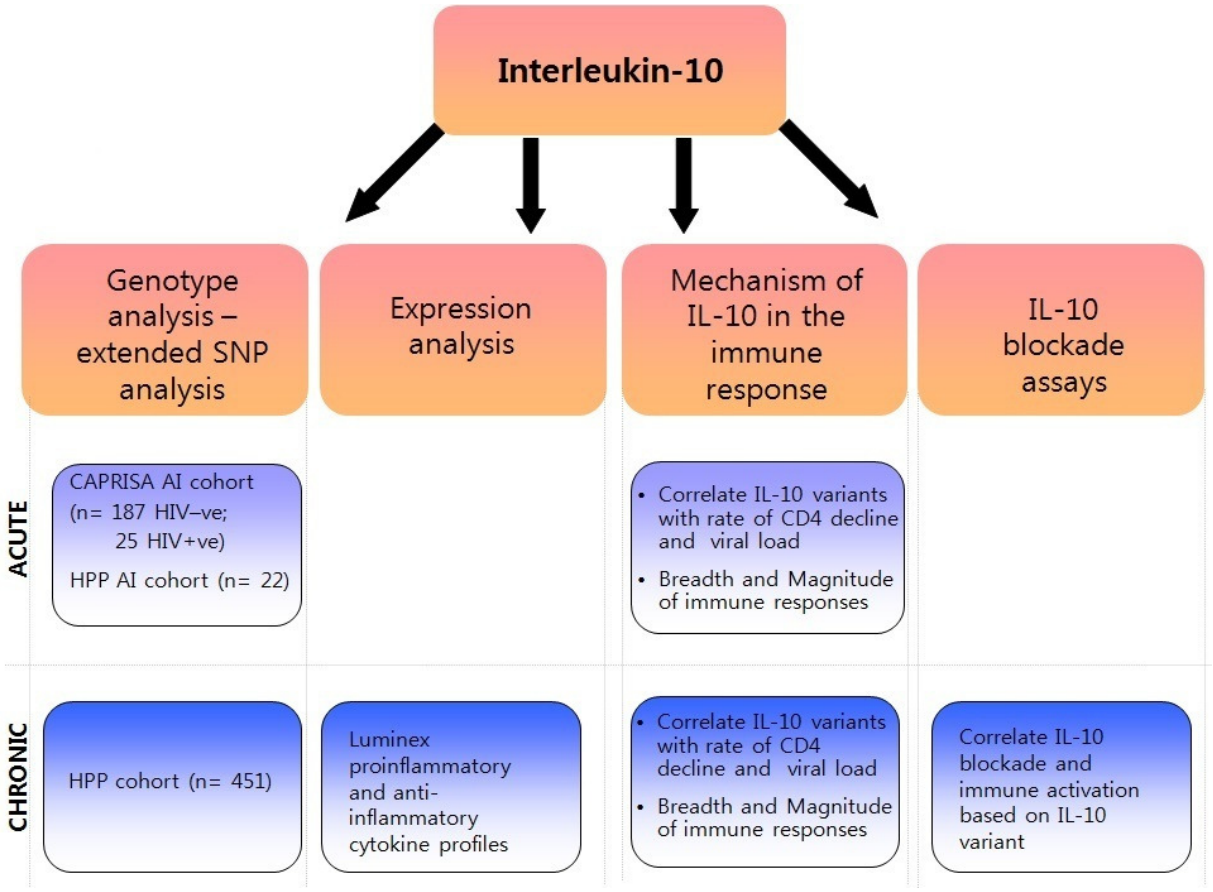

Figure 1.5.1 Outline of project strategy

Chapter 2

Genotype analysis of *IL-10* promoter polymorphisms, and association with biomarkers of HIV-1 infection

Chapter 2: Genotype analysis of *IL-10* promoter polymorphisms, and association with biomarkers of HIV-1 infection

INTRODUCTION

There is an uneven distribution of HIV globally, with two-thirds of the world's infected population found in sub-Saharan Africa. There are multiple factors which contribute to this uneven distribution of HIV infection, including socio-economic factors and biological factors. Some examples of socio-economic factors include poverty, nutrition, and health-care. Examples of biological factors include the strain or subtype of the virus present in a specific geographical region, concomitant infections and host genetic factors. As HIV replication takes place within the host's cells, it utilises host proteins, enzymes, amino acids, ribosomes and energy. Therefore if certain host genetic factors influenced any part of the HIV replication cycle, this would affect susceptibility to HIV infection and the rate of disease progression. It has been demonstrated that different host genetic factors may influence different stages of HIV infection, such as HIV susceptibility, acute or chronic infection (Gao et al., 2005, Naicker et al., 2009, Shin et al., 2000). Thus understanding the effect of host genetic factors which may influence different stages of HIV infection is essential.

Allelic frequencies in one geographic or ethnic population, may not always translate to another geographic or ethnic population. These differences can be attributed to the age of the population and diverse mutational forces that shape human evolution (Donfack et al., 2006). Systematic and evolutionary forces, such as natural selection, random genetic drift, migration and mutation shape the process of evolution (Snustad, 1999). Therefore allelic frequencies of certain polymorphisms may not always translate from one geographic population to the next.

Therefore ethnic diversity of a population may possibly play a role in the distribution of host genetic factors that restrict HIV susceptibility and pathogenesis.

An example of the geographical differences of host genetic factors that influence HIV infection and pathogenesis is the mutation found in the chemokine binding co-receptor CCR5, i.e. the CCR5Δ32 variant (Dean et al., 1996, Kostrikis et al., 1999, Paxton et al., 1996). This was the first host restriction factor shown to effectively block against HIV-1 infection. HIV requires two surface receptors to enter a cell, i.e. CD4 and the chemokine binding receptor that is most commonly used is the CCR5 co-receptor. The CCR5Δ32 mutation is a 32 base pair deletion in the CCR5 gene. If an individual is homozygous for this mutation, then the CCR5 co-receptor is non-functional and this mutation is strongly associated with protection against HIV-1 infection (Kostrikis et al., 1999). However, the frequency of this mutation differs geographically and is very rare among Africans. It has been shown that the frequency of the CCR5Δ32 mutation is higher among Northern Europeans, such as the Swedish population, decreasing geographically south in the British, German, French, Italian, Greek and Turkish populations (Stephens et al., 1998).

The human leukocyte antigen (HLA) locus is a highly polymorphic region which has also been implicated as an HIV host genetic restriction factor. HLA genes are part of the MHC locus. The function of HLA class I molecules is to present pathogen-derived peptides on the surface of infected cells for $CD8^+$ T lymphocyte recognition. Different HLA genotypes have been associated with a difference in immune responses, HIV-1 susceptibility to infection and

disease progression (Fellay et al., 2007, Kiepiela et al., 2004, Limou et al., 2009). Similarly, the distribution (or population frequencies) of these HLA genes varies geographically.

IL-10 is a potent, anti-inflammatory Th2 cytokine, which is involved in regulating the immune response. Previous genetic studies have focused on the three classic proximal promoter SNPs. These SNPs are found at positions -592 (a C to A transversion); -819 (a C to T transition); and -1082 an A to G transition. The -592 and -819 SNPs are in complete linkage disequilibrium, so the mutation at one position is always present with the mutation at the other position. Three SNPs found in the distal region have recently been identified, they are found at positions -2763 (C to A transversion); -2849 (G to A transition); and -3575 (T to A transversion).

The *IL-10* -592A variant has shown to be associated with an increased susceptibility to HIV-1 infection, and an acceleration to AIDS among Caucasians (Oleksyk et al., 2009, Shin et al., 2000, Vasilescu et al., 2003). In a genetic study performed in an African Zimbabwean cohort, it was found that carriers of the -1082G allele showed that survival was doubled (Erikstrup et al., 2007). We have recently shown in a cohort of high-risk black South African women that the -1082AA and -592AA genotypes were associated with an increased risk of HIV infection (Naicker et al., 2009). Once infected, these genotypes associated with high viral load and low $CD4^+$ T cell counts during the acute phase of infection. This suggests that the effect of *IL-10* polymorphisms may depend on the phase of infection.

In this part of the study we wanted to determine, in South African cohorts, the allele and genotypes frequencies of three *IL-10* promoter polymorphisms (-592, -1082 and -3575) shown to be associated with HIV-1 susceptibility and pathogenesis. We expanded this study to

include high risk HIV-negative women, acutely HIV-1 infected individuals and chronically HIV-1 infected individuals. This would give us an indication of the role of *IL-10* polymorphisms during different stages of HIV infection. Our project strategy included association analyses for viral load, $CD4^+$ T cell counts, and CD4 decline. These analyses would allow us to investigate the role of a previously identified ARG in mediating differential susceptibility and pathogenesis in high risk women, and antiretroviral naïve individuals at different stages of HIV-1 infection. Understanding the protective mechanisms against HIV-1 infection may lead to the design and development of therapeutic interventions and possible vaccine strategies.

2.2 MATERIALS AND METHODS

2.2.1 STUDY POPULATION

This study population consisted of three different cohorts (See Figure 2.2.1). These groups were the CAPRISA (Centre for the AIDS Programme of Research in South Africa) Acute Infection cohort, the HPP (HIV Pathogenesis Programme) Acute Infection cohort, and the HPP Sinikithemba Chronic Infection cohort.

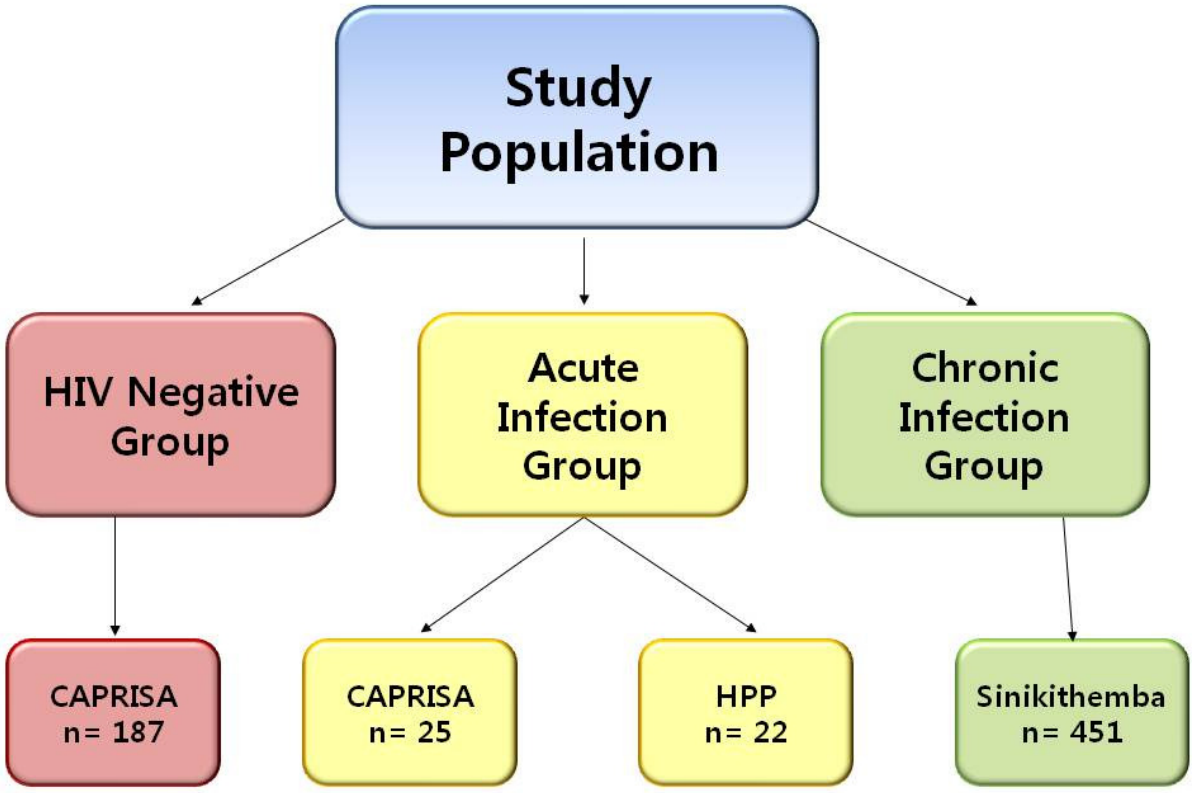

Figure 2.2.1 Breakdown of study population. Three different cohorts were studied at different stages of infection.

Figure 2.2.2 shows the breakdown of the CAPRISA Acute Infection cohort. The CAPRISA Acute Infection (CAI) study is a longitudinal cohort study on viral set point and clinical progression in HIV-1 subtype C infection (van Loggerenberg et al., 2008). This cohort was established in Durban, South Africa in 2004. The study enrolled 245 high risk HIV negative women who were screened monthly for HIV infection using two rapid antibody tests (Determine, Abbott Laboratories, Illinois, IL, USA; and Capillus, Trinity Biotech, Jamestown, NY, USA). Negative or indeterminate samples were subjected to pooled plasma PCR testing, and positive pools were deconstructed and individually tested (COBAS Ampliscreen^{TM} HIV-1 Test, Roche Diagnostics, Indianapolis, IN, USA). HIV-1 infection was further confirmed in RNA positive samples with a positive HIV enzyme immunoassay test (Enzygost, Siemens Healthcare Diagnostics, Eschborn, Germany), and a diagnostic nucleic acid test (Roche Diagnostics, Indianapolis, IN, USA). Women from this HIV-negative cohort and other seroincidence cohorts were enrolled into phase II of the study if they seroconverted during follow-up. $CD4^+$ T cell counts were determined by flow cytometry (Becton Dickinson, San Jose, CA, USA). Plasma viral loads and $CD4^+$ T cells counts were performed at various time points post-infection. As this study follows individuals from before seroconversion and early seroconversion, this allowed us to study susceptibility from HIV-negative individuals as well as progression from acute infection to early chronic infection.

The HPP Acute Infection (HAI) cohort had 33 participants at the time of this study (see Figure 2.2.3). This study was initiated in March 2007, in Durban, South Africa (Radebe et al., 2011). All participants were identified by screening at three voluntary counseling and testing centres at St Mary's hospital, McCord hospital and Prince Mshiyeni hospital in Durban, South Africa. Participants that tested negative by Rapid HIV-1 tests (Bioline, Standard Diagnostics; and

Sense, Hitech Healthcare) were tested for being HIV-1 RNA positive; negative HIV-1 enzyme immunoassay (SD HIV1/2 enzyme-linked immunosorbent assay [ELISA] 3.0, Standard Diagnostics), and a negative or indeterminate Western blot (Genetic System, Bio-Rad, Hercules, CA, USA). Once identified as acutely infected, participants are followed longitudinally. Viral load measurements were performed using Roche Amplicor version 1.5 assay or Cobas Taqman HIV-1 test, and $CD4^+$ T cell counts were measured by Tru-Count technology and flow cytometry (Becton Dickinson, San Jose, CA, USA).

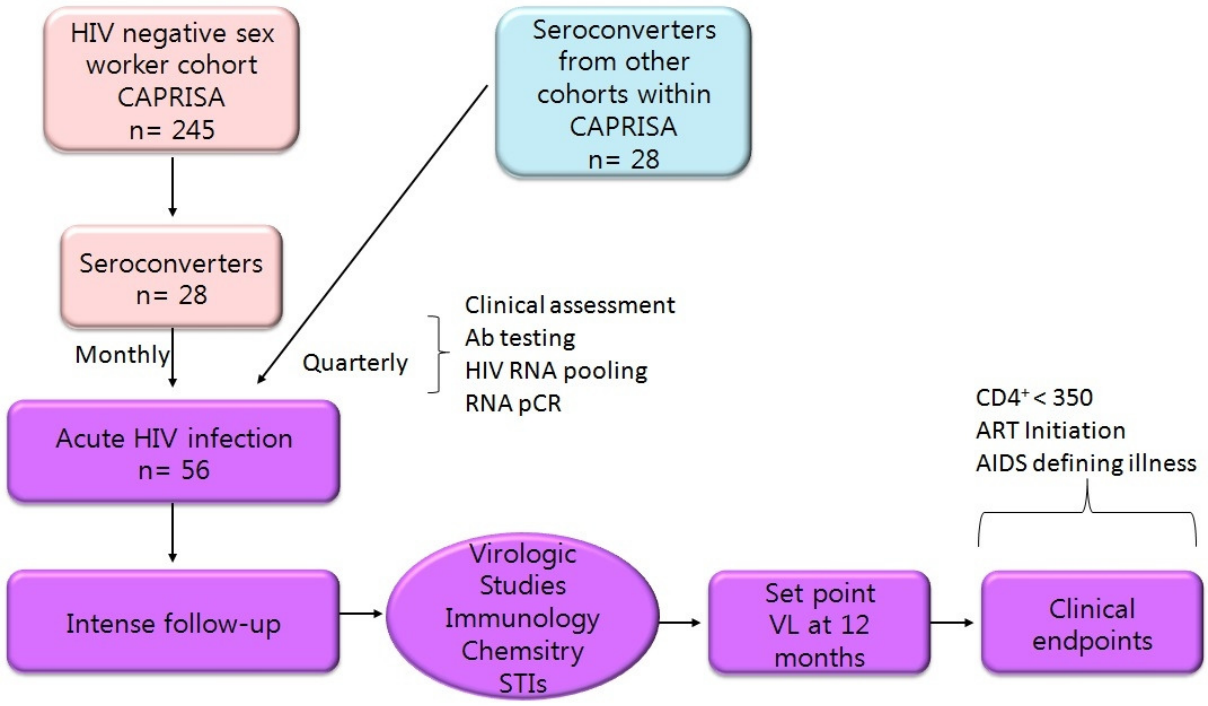

Figure 2.2.2 The breakdown of the CAPRISA Acute Infection Cohort. This cohort was initiated in 2004, in Durban, South Africa. The Acute Infection Cohort has followed individuals at high risk for HIV infection from when they were HIV-negative until seroconversion. Various measurements and follow-up is done following seroconversion.

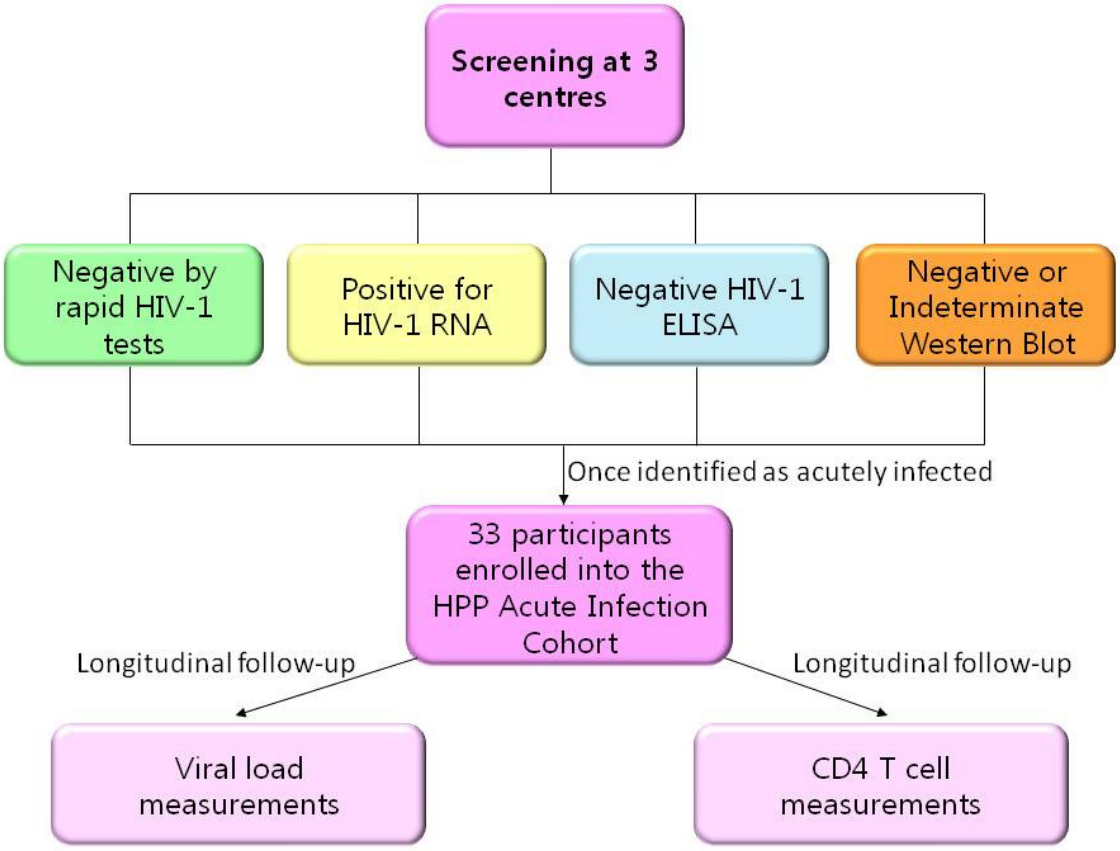

Figure 2.2.3 An overview of the HPP Acute Infection Cohort. The HPP Acute Infection cohort was initiated in March 2007, in Durban, South Africa. Once individuals are identified as acutely infected they are enrolled into the study with longitudinal follow-up.

The HPP Sinikithemba (SK) cohort is a longitudinal study of chronic HIV infection (see Figure 2.2.4). This cohort was established in Durban, South Africa in August 2003, and participants were enrolled into the study until June 2006 (Kiepiela et al., 2007). This study group consists of 451 antiretroviral naïve, chronically HIV-1 subtype C-infected adults. The Sinikithemba cohort is based at McCord Hospital in Durban, South Africa. $CD4^+$ T cell counts and plasma viral loads were performed routinely for study participants, including the baseline time point of study entry. $CD4^+$ T-cell counts were measured by flow cytometry (Becton Dickinson, San Jose, CA, USA) while plasma viral loads were measured using the Roche

Amplicor version 1.5 assay (Roche Diagnostics, Indianapolis, IN, USA). $CD4^+$ T cell counts were performed at 3-month intervals and plasma viral loads were performed at 6-month intervals. The number and magnitude of HIV peptides targeted by CTL were measured at baseline by interferon-γ (IFN-γ) enzyme-linked immunosorbent (ELISPOT) assay using a panel of 410 overlapping peptides spanning the HIV-1 subtype C (HIV-1C) proteome.

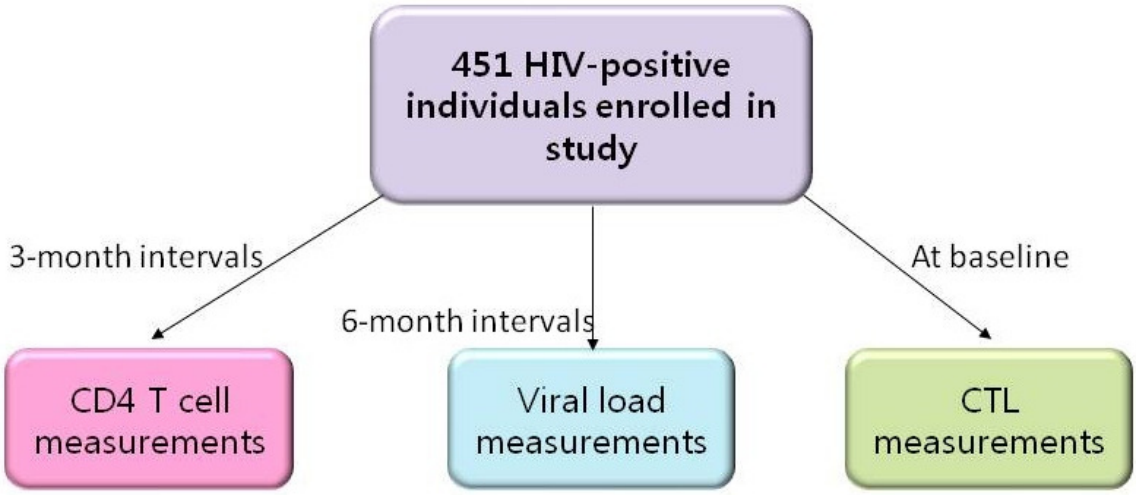

Figure 2.2.4 An overview of the Sinikithemba Chronic Infection cohort. This cohort was initiated in August 2003, in Durban, South Africa. Participants were enrolled into the cohort after they tested positive for HIV infection. Longitudinal measurements of biomarkers of HIV infection were done.

At the time of assessment, the total number of individuals enrolled in the CAI cohort was 222. Of this total, 3 individuals were later found to have been HIV-positive at enrollment, and were excluded from the cohort. Of the remaining 219, 212 samples were available for genotype assessment and were included in this study. A total of 25 individuals acquired HIV during the follow-up period. At the time of assessment, the total number of individuals included in the

HAI cohort was 33. Of this total, 22 samples were available for genotype assessment and were included in this study. At the time of assessment, the total number of individuals included in the SK cohort was 451. All 451 samples were available for genotype assessment, and were included in this study. Overall, 685 individuals were included in this study, 498 HIV-positive individuals and 187 HIV-negative individuals.

2.2.2 SAMPLE PREPARATION

Participants enrolled in these studies provided blood samples at scheduled visits and time points. At each visit blood samples were collected by venipuncture into EDTA tubes. Samples from the CAI cohort were prepared to separate cellular components from plasma, before DNA extraction was performed. DNA was extracted from whole blood samples from the HAI and SK cohorts.

For CAI samples, cellular components were separated from plasma. This was done by centrifuging whole blood at 2,500 x g for 10 minutes at room temperature, using a Jouan MR23i centrifuge. After whole blood is centrifuged, it separates into three layers based on their density (see Figure 2.2.5). After centrifugation the erythrocytes (red blood cells) settle at the bottom of the tube, the intermediate layer follows consisting of leukocytes (white blood cells) and thrombocytes (platelets), and the uppermost layer is the plasma. The intermediate layer is known as the buffy coat, from which DNA extraction is performed.

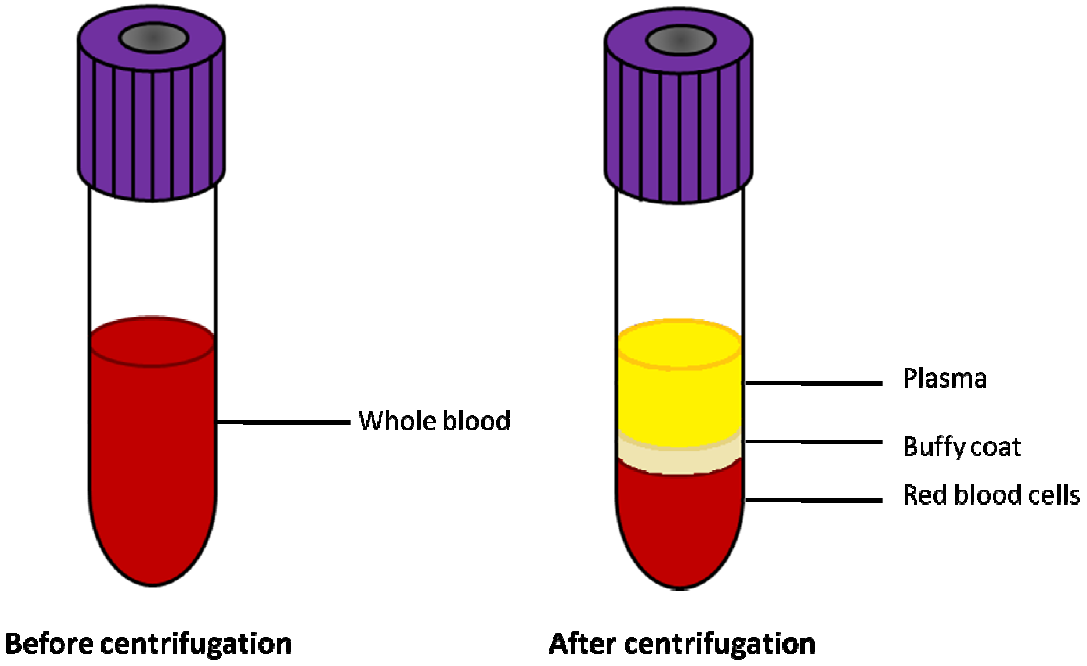

Figure 2.2.5 Separation of whole blood. After whole blood is centrifuged, it separates into three layers, i.e. bottom layer of red blood cells, middle layer of buffy coat and top layer of plasma.

Buffy coats were carefully removed from EDTA tubes using fine tip plastic Pasteur pipettes (Copan, Murrieta, CA, USA) and placed into clean 1.5 ml Eppendorf tubes (Axygen, Union City, CA, USA). Buffy coats were stored at -80^{o}C until processed for DNA extraction. Excess red blood cells were removed from the buffy coat using RBC Lysis Solution (QIAGEN, Valencia, CA, USA), to avoid downstream inhibition of polymerase chain reaction (PCR). 1 ml of RBC Lysis Solution was added to each sample, which was then left at room temperature for 10 minutes. To allow for lysis of red blood cells samples were periodically inverted. After 10 minutes, samples were centrifuged at 4,000 rpm for 10 minutes at room temperature, using the Jouan A14 microcentrifuge. After centrifugation the buffy coat forms a

pellet at the bottom of the microcentrifuge tube. The supernatant contains lysed red blood cells, which were removed from the microcentrifuge tube, leaving behind 200 µl to resuspend the buffy coat pellet. This amount of sample was sufficient for DNA extraction.

2.2.3 DNA EXTRACTION

DNA was extracted from samples from the CAI cohort using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA). DNA extraction was performed on 200 µl of buffy coat following the manufacturer's protocol. If samples were less than 200 µl, then PBS was used to bring the starting volume up to 200 µl. If samples were more than 200 µl, then the amount of lysis buffer and other reagents were increased proportionally. An overview of this protocol is shown in Figure 2.2.6. Briefly, Protease (or Proteinase K) was added to the sample to lyse and expose the contents of the cells. Buffers were added to the samples which enabled the DNA to bind to the spin column provided in the kit. During centrifugation, the DNA from samples bound to the membrane of the spin column and cellular debris flowed through the membrane and collected in a collection tube which was then discarded. The salt and pH conditions of the lysate prevents cellular debris such as protein and other contaminants from binding to the membrane. Thereafter, two wash steps allow for the removal of residual contaminants. Finally, the purified DNA is eluted using Buffer AE, allowing for long-term storage of DNA at -80^{o}C for future use.

DNA from samples from the HAI and SK cohorts were extracted using the Gentra Puregene DNA extraction kit (QIAGEN, Valencia, CA, USA). DNA was extracted from 300 µl of whole blood, following the manufacturer's protocol (see Figure 2.2.6). Briefly, RBC Lysis

Solution was used to lyse excess red blood cells, which may inhibit downstream PCR reactions. After incubating in RBC Lysis Solution for 1 minute at room temperature, samples were centrifuged for 20 seconds at 13,000 x g to pellet the white blood cells. The supernatant, containing the lysed red blood cells, was then carefully discarded leaving behind approximately 10 µl of residual liquid to resuspend the pellet. The Cell Lysis Solution was added to lyse the cells, followed by the Protein Precipitation Solution to the lysed cells. After centrifuging, the protein debris formed a pellet at the bottom of the tube, and the supernatant containing the DNA was pipetted into a clean tube containing isopropanol. After inverting this mix, the DNA became visible as threads or a clump. This was then centrifuged, and the supernatant discarded. The DNA pellet was then washed using 70% ethanol. After centrifuging again, the supernatant was discarded leaving behind the DNA pellet, which was then hydrated using the DNA Hydration solution. Finally, the DNA was incubated at 65^{o}C for 5 minutes, and then stored at -80^{o}C for future use.

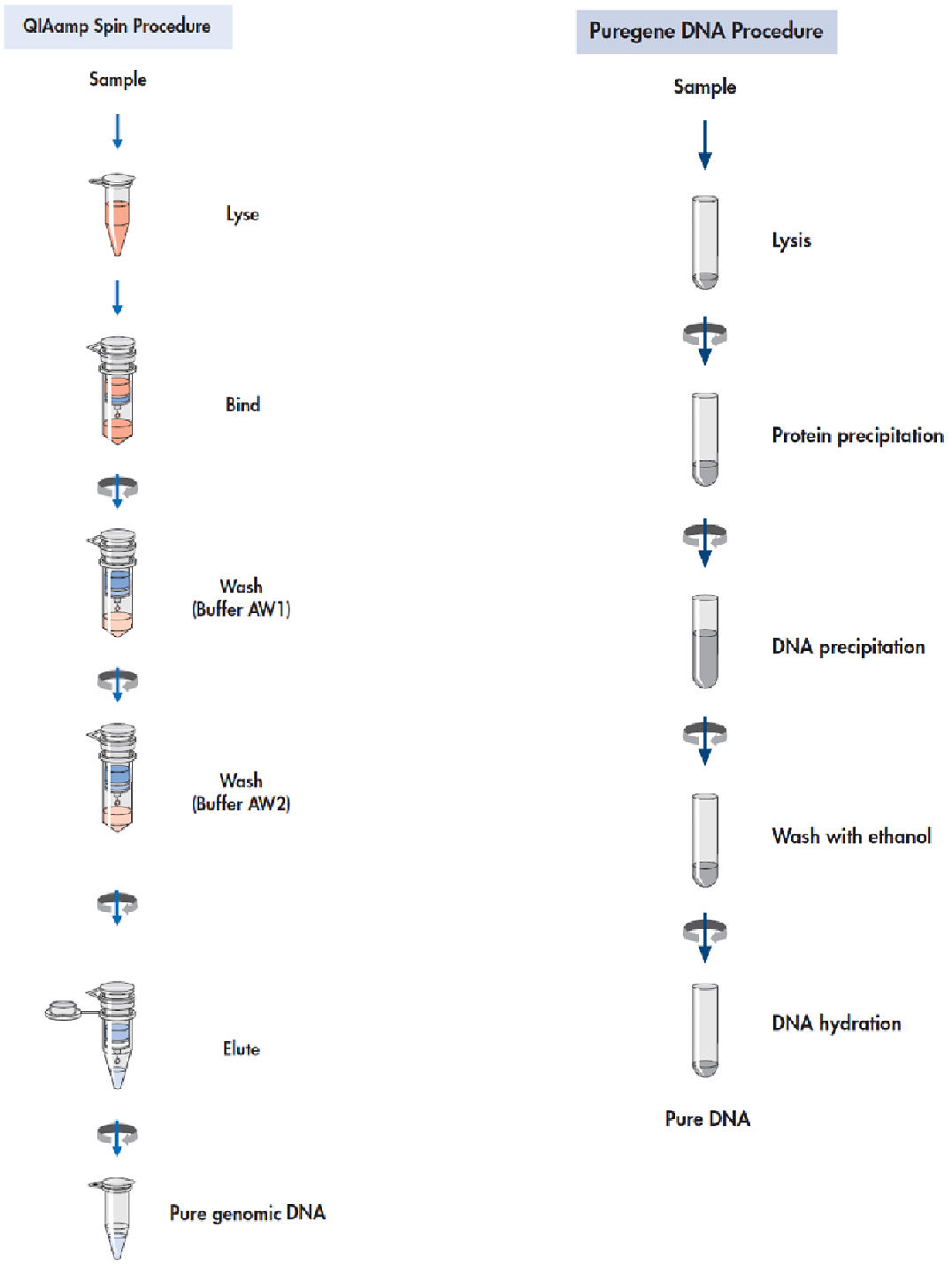

Figure 2.2.6 Overview of protocols used for DNA extraction (taken from QIAGEN)**.** The QIAmp Spin Procedure was used to extract DNA from CAPRISA Acute Infection Cohort. The Puregene DNA procedure was used to extract DNA from samples from both the HPP Acute Infection cohort and the Sinikithemba chronic infection cohort.

All DNA samples were quantified using the NanoDrop (Thermo Scientific, Waltham, MA, USA). Blank measurements were made using Buffer AE (elution buffer) for samples extracted using the QIAamp DNA Blood Mini Kit, and DNA Hydration Solution for samples extracted using the Gentra Puregene DNA extraction kit. DNA samples were quantified using 1.5 µl of sample which was placed on the NanoDrop sample platform, and a measurement was then taken and recorded.

2.2.4 GENOTYPE ASSESSMENT USING TAQMAN SNP GENOTYPING

Three IL-10 promoter polymorphisms were genotyped in our study cohort. These SNPs are found at positions -592 (rs1800872), -1082 (rs1800896) and -3575 (rs1800890). Genotype assessment was done on our study cohort using TaqMan SNP Genotyping assays (Applied Biosystems, Life Technologies, Grand Island, NY, USA). Predesigned primers and probes were used to genotype all three SNPs analysed in this study.

An overview of the TaqMan Genotyping assay is shown in Figure 2.2.7. The TaqMan genotyping assay consists of a single tube per SNP. Each assay contains two primers which amplify the polymorphic sequence of interest, and two TaqMan MBG (Minor groove binder) probes which distinguish between the two alleles, i.e. the wild-type and the polymorphism.

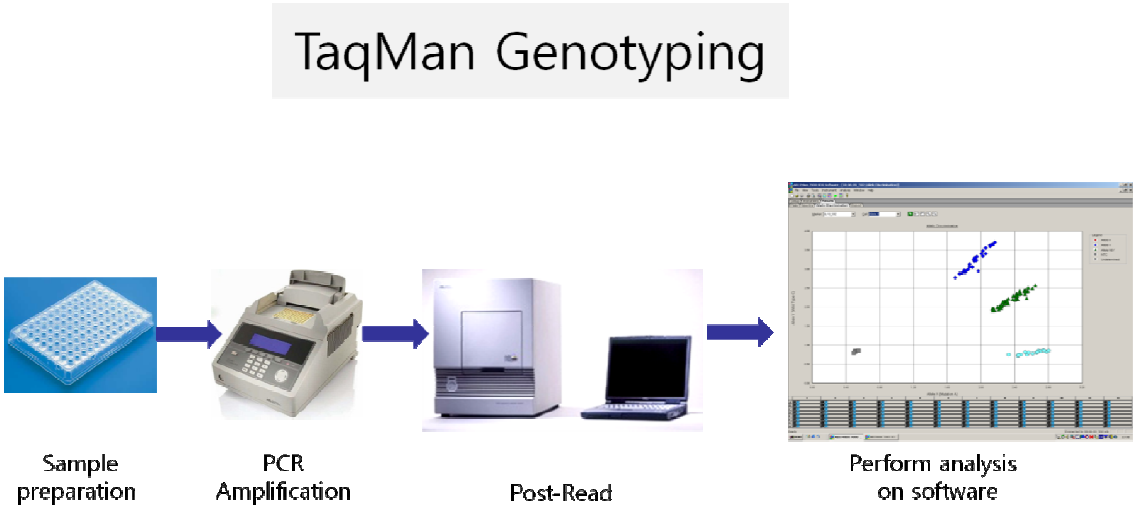

Figure 2.2.7 An overview of the Taqman Genotyping assay (images taken from (Abgene, Biosystems)). Samples are prepared and reactions are arranged on a 96-well plate. PCR amplification is performed and samples are subjected to a post-read on the ABI Prism 7000 Sequence detection system. Analysis of data is performed on the ABI software and genotypes are assigned to samples.

The TaqMan SNP genotyping assay requires 1-20 ng of genomic DNA per reaction. All DNA samples genotyped, were diluted down to 20 ng/µl of DNA with sterile distilled water. If the sample was less than 20 ng/µl after quantification, then those samples were used as is, provided the purity of the sample was adequate, i.e. the ratio of the A260/A280 was between 1.7-1.9. Each reaction contained: 12.5 µl of TaqMan Universal PCR Master Mix, 0.625 µl of genotyping assay probe and primer mix, 1 µl of DNA (at concentration between 1-20 ng/µl), and 10.875 µl of sterile distilled water to make up the reaction volume to a total of 25 µl per well. Samples were prepared on a 96-well plate. All reaction plates contained negative controls, i.e. sterile distilled water was used instead of DNA template, and these negative controls are referred to as non-template controls (NTC). All reaction plates contained positive

controls as well. These were samples from the CAPRISA Acute Infection cohort that produced positive results previously by TaqMan genotyping and were further confirmed by sequencing. Each plate also contained random duplicates of test samples. An example of a typical plate layout is shown in Table 2.2.1.

The thermal cycling conditions used were from the standard protocol provided in the manufacturer's protocol (see Table 2.2.2). All reactions were run on a GeneAmp PCR system 9700 (Applied Biosystems, Life Technologies, Grand Island, NY, USA).

Table 2.2 An example of a plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	SK061	SK069	SK076	SK084	SK091	SK099	SK107	SK114	SK121	SK129	SK137	**SK062**
B	SK062	SK070	SK077	SK085	SK092	SK100	SK108	SK115	SK122	SK130	SK138	**0028**
C	SK063	SK071	SK078	SK086	SK093	SK101	**H_2O**	SK116	SK123	SK131	SK139	**SK073**
D	SK064	**H_2O**	SK079	SK087	SK094	SK102	SK109	SK117	SK124	SK132	SK140	**SK078**
E	SK065	SK072	SK080	SK088	SK095	SK103	SK110	SK118	SK125	SK133	SK141	**SK098**
F	SK066	SK073	SK081	**H_2O**	SK096	SK104	SK111	**H_2O**	SK126	SK134	SK142	**0048**
G	SK067	SK074	SK082	SK089	SK097	SK105	SK112	SK119	SK127	SK135	SK143	**SK119**
H	SK068	SK075	SK083	SK090	SK098	SK106	SK113	SK120	SK128	SK136	**0001**	**SK136**

1. Distilled water, indicated by blue blocks, were used as non-template controls (NTC)
2. Positive controls were included, indicated by green blocks
3. Random duplicates are indicated by orange blocks

Table 2.2.2 PCR conditions used for TaqMan SNP Genotyping

Step	AmpliTaq Gold Enzyme Activation	PCR (40 cycles)		HOLD
		Denature	Anneal/Extend	
Time	10 minutes	15 seconds	1 minute	∞
Temperature	95°C	92°C	60°C	4°C

During PCR amplification the 5' nuclease process occurs, this is shown in Figure 2.2.8. Each probe anneals to its complementary sequence of template DNA, between the forward and reverse primer sites. The TaqMan MGB probes contain a reporter dye at the 5' end of the probe. The reporter dye is either VIC or FAM, which are linked to either the probe for Allele 1 or Allele 2 respectively. The MGB is found at the 3' end of the probe, which allows probes of shorter length to stably hybridise to the complementary DNA sequence. This results in the melting temperature between the matched and mismatched probes, resulting in robust allelic discrimination. The probes also contain a nonfluorescent quencher (NFQ) at the 3' end. This prevents fluorescence of the dye from unhybridised probes. The AmpliTaq Gold DNA polymerase extends the primers which are bound to the template DNA. Cleaving of probes only occurs when they are hybridised to the target DNA sequence. When the probes are cleaved the reporter dye is separated from the quencher dye, resulting in fluorescence of the reporter dye VIC or FAM. When probes that have hybridised to the complementary sequence are cleaved, an increase in fluorescence occurs. Therefore, fluorescence resulting from PCR amplification indicates which alleles are present in the sample. For example, when there is an increase in the VIC dye, then the sample is homozygous for Allele 1, when there is an increase

in FAM-dye, then the sample is homozygous for Allele 2, when both the VIC and FAM dyes fluoresce, then the sample is heterozygous for both alleles.

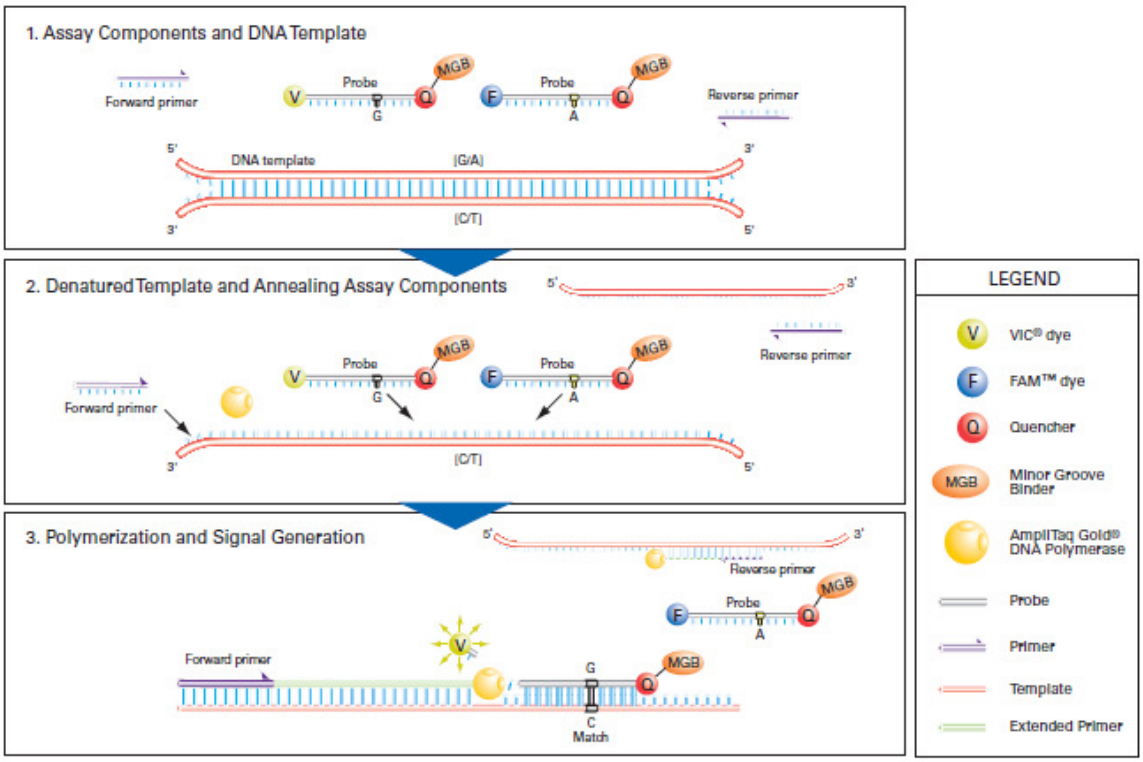

Figure 2.2.8 Overview of the 5' Nuclease assay process (taken from ABI). Two primers specifically amplify the region of interest, while the two probes with reporter dyes fluoresce based on the allele present in the target sequence. After the probe binds to the region of interest, the AmpliTaq Gold DNA Polymerase cleaves the quencher dye, causing the reporter dye to increase in fluorescence, thus allowing for allelic discrimination based on increase in fluorescent signal.

Allelic discrimination plate reads and analyses were performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Life Technologies, Grand Island, NY,

USA). After PCR amplification, the endpoint plate read was performed. The Sequence Detection System (SDS) software plotted the fluorescence measurements based on signals from each well, i.e. each sample. Based on the fluorescence signal, alleles are identified for each sample. An example of the plotted fluorescence signals is shown in Figure 2.2.9. This example shows the fluorescence plot for the -592 mutation. The fluorescence signal for the mutation -592A is plotted on the x-axis, while the fluorescence signal for the wild-type allele -592C is plotted on the y-axis. Here, the three genotypic groups are represented by different shapes and colours. The genotypes group separately, allowing for allelic discrimination. The blue diamonds represent individuals homozygous for the wild-type allele, i.e. the -592CC genotype, the green triangles represent individuals heterozygous for both alleles, i.e. -592CA and the red circles represent individuals homozygous for the polymorphism, i.e. -592AA. The grey squares are the NTCs. The genotypes are reported in table form as well for easy allelic discrimination.

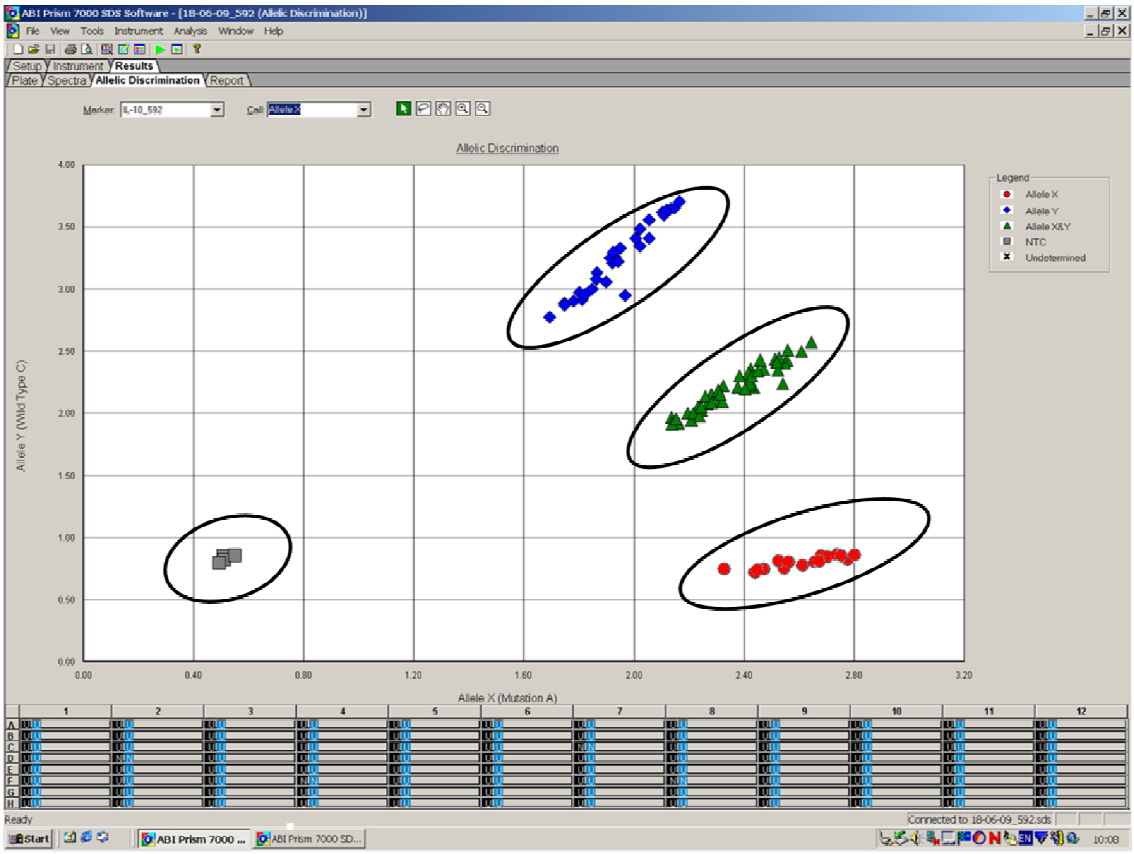

Figure 2.2.4 An example of a fluorescence plot used for allelic discrimination. The three genotypes group separately allowing for easy allelic discrimination. The NTCs are represented by the grey squares.

2.2.5 STATISTICAL ANALYSIS

Various methods of statistical analysis were used. The association between different *IL-10* variants and plasma viral load, $CD4^+$ T cell count, the rate of CD4 decline and the magnitude and breadth of immune responses were analysed.

The allelic frequencies of the *IL-10* variants, in the total study population as well as subpopulations included in different analyses, were confirmed to be in Hardy-Weinberg equilibrium using the χ^2 (Chi-square) test. To determine the association between *IL-10*

variants and either plasma viral load or $CD4^+$ T cell count, we used the Kruskal-Wallis test. The haplotypes were generated using an unpublished program from the Head of the Bioinformatics Section of the Frederick National Laboratory. Haplotypes were generated using the using the expectation maximization (EM) algorithm (Excoffier and Slatkin, 1995). To determine if there was an association between *IL-10* haplotypes and either viral load or $CD4^+$ T cell count, we used the Wilcoxon rank sums test. The rate of CD4 decline was measured over a period of 24 months, the association between *IL-10* variants and CD4 decline was estimated using the multivariate mixed effects models. To determine the association between *IL-10* variants and the breadth and magnitude of immune responses, the Kruskal-Wallis test was used.

2.3 RESULTS

2.3.1 CLINICAL DETAILS OF PARTICIPANTS INCLUDED IN STUDY

Demographics of the three cohorts included in this study are shown in Table 2.3.1. All participants in the CAI cohort underwent monthly screening for HIV-1 infection. Of the 212 individuals included in this study, 25 had acquired HIV during the two-year follow-up period and were enrolled into phase II of the study. The remaining 187 HIV-negative individuals did not acquire HIV infection during the follow up period and were disenrolled from the study after two years. The median age of individuals in the CAI cohort was 36 years, and all individuals were female. The median age of individuals in the HAI cohort was 32 years and 52% of individuals in this study were female. The median age of individuals in the SK cohort was 31 years and 82% of individuals in this cohort were female.

Table 2.3.1 Demographics of individuals included in study

Cohort	Number of Individuals	Median Age (years)	Female (%)
CAPRISA Acute Infection	212	36 (18-58)	100
HPP Acute Infection	22	32 (21-66)	52
HPP Sinikithemba	451	31 (27 – 37)	82

2.3.2 DNA EXTRACTION AND QUANTIFICATION

DNA was extracted from the buffy coat of 212 individuals from the CAI cohort using the QIAamp DNA Blood Mini Kit (QIAGEN). DNA was quantified using the Nanodrop and the concentration ranged from 5-150 ng/µl. DNA was extracted from both the HAI and SK cohorts routinely using the Gentra Puregene DNA Extraction kit (QIAGEN). DNA was quantified using the Nanodrop and the concentration ranged from 9-2,000 ng/µl. All samples had a purity of $\pm$ 1.8 (A260/A280 ratio). The amount of DNA required for the genotyping was between 1-20 ng. Samples with concentrations lower than 20 ng/µl were used as is, and samples with concentrations more than 20 ng/µl were diluted down to 20 ng/µl using sterile distilled water.

2.3.3 *IL-10* GENOTYPE AND HAPLOTYPE FREQUENCIES AND -3575 DISTRIBUTION

Previously described *IL-10* promoter polymorphisms were present in this study population. The overall allele frequencies of the three SNPs studied are shown in Table 2.3.2. The -592 and -1082 SNPs were studied previously (Naicker et al., 2009). Genotype data for all individuals included in this study are found in Appendix 1. Previous studies determined the frequency of these alleles in different ethnic populations. The frequency of the -592A allele was 0.24 in Caucasians, 0.4 in African Americans, 0.33 in Hispanics, and 0.6 in Asians (Shin et al., 2000). The frequency of the 1082G allele was 0.5 in Dutch Caucasians, 0.35 (Italian cohort), 0.47 (Netherlands cohort), and 0.28 (Indian cohort) (Bagnoli et al., 2007, Chatterjee et al., 2009, de Jong et al., 2002, Keijser et al., 2009). The frequency of the -3575A allele was 0.43 in Caucasians, 0.29 (Spanish cohort), 0.29 in Afro-Brazilians, 0.33 in Euro-Brazilians,

0.4 in Dutch, 0.3 in Brazilians (de Jong et al., 2002, Domingo-Domenech et al., 2007, Moraes et al., 2003).

Haplotypes were generated for individuals genotypes for all three polymorphic regions of interest. Haplotypes were then generated based on these three genotypes, and assigned to individuals based on probabilities. If an individual was homozygous for a SNP at all three regions, then that individual would only have one haplotype. However, if an individual was heterozygous at any of these three polymorphic sites then there would be a possibility of having two haplotypes based on the highest probability score (a score of 0.89 or greater). Table 2.3.3 below shows the percentage of individuals with a specific haplotype, shown separately, i.e. based on whether an individual had the haplotype or not. In this study group, there was a possibility of 7 haplotype groups: CAT, AAT, CGA, CGT, CAA, AGT and AGA.

Table 2.3.2 Allele frequencies of all three SNPs in study cohorts

Cohort	-592C (Wild-type)	-592A (Mutation)	-1082A (Wild-type)	-1082G (Mutation)	-3575T (Wild-type)	-3575A (Mutation)
CAPRISA Acute Infection	*	*	*	*	0.8	0.2
HPP Acute Infection	0.73	0.27	0.59	0.41	0.68	0.32
HPP Sinikithemba	0.69	0.31	0.68	0.32	0.77	0.23

*These mutations were previously studied and allele frequencies were described in my preceding research (Naicker et al., 2009)

Table 2.3.3 Haplotype frequencies of all three study cohorts

Cohort	AAT	AGA	AGT	CAA	CAT	CGA	CGT
CAPRISA Acute Infection	0.29	0.0003	0.03	0.02	0.34	0.21	0.12
HPP Acute Infection	0.24	0	0	0.02	0.27	0.32	0.15
HPP Sinikithemba	0.34	0	0	0.02	0.32	0.23	0.11

2.3.4 ASSOCIATION OF *IL-10* -3575 GENOTYPE WITH DISTRIBUTION AND TIME TO INFECTION

The CAI cohort, was the only cohort in this study that followed individuals from when they were HIV-negative until seroconversion. Therefore, for this analysis we only included the CAI as we had data for both HIV-negative and HIV-positive individuals. As they were also followed up until seroconversion, we were able to analyse if the *IL-10* -3575 SNP had any association with HIV-status or time to infection.

For this part of the analysis, 212 individuals were genotyped for the -3575 SNP using TaqMan Genotyping assays (Applied Biosystems). The distribution of the -3575 genotypes is shown in Figure 2.3.1. The blue bars indicate HIV-negative individuals and the red bars indicate HIV-positive individuals. The number of individuals is indicated within bars and the percentage of each genotype according to HIV-status is also indicated. The Fisher's exact test was used to determine if there was a significant association between -3575 genotype and HIV-status. The distribution of HIV-positive individuals was TT: 13.6% (18/132), TA: 6.8% (5/74) and AA: 33.3% (2/6) (p= 0.08). Therefore, looking at the -3575 mutation on its own, there was no significant association between -3575 genotype and HIV status, however there seemed to be a trend towards an association.

We also wanted to investigate if there was an association between CAI haplotype and HIV acquisition (Table 2.3.4). All participants that were followed since they were HIV negative were included in this analysis. Time to HIV acquisition was calculated as time from enrolment into the HIV negative cohort to estimated date of infection. A proportional hazards model was

fitted to time-to-HIV acquisition with haplotype. We did not find any significant association between any CAI haplotype and HIV acquisition. As the AGA and CGT haplotypes had zero HIV-positive individuals, we used the Fisher's exact test to calculate the *p*- value. The AGA haplotype was not significantly associated with HIV acquisition (p= 1.00), however, we found that the CGT haplotype was significantly associated with HIV acquisition (p= 0.003). There were zero HIV-positive individuals with the CGT haplotype.

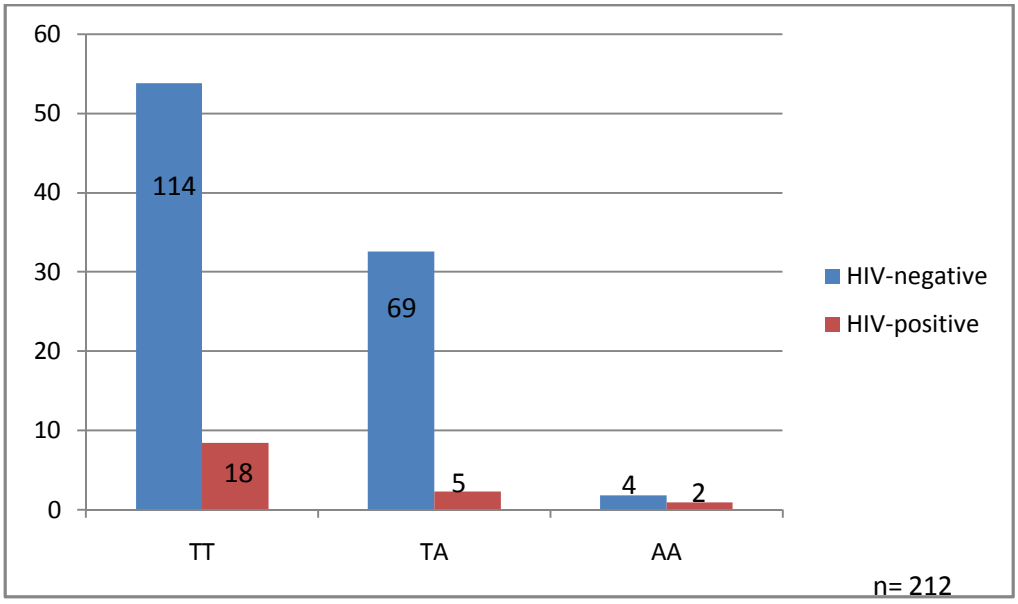

Figure 2.3.1 The percent distribution of -3575 genotype with HIV-status. The blue bars indicate the HIV-negative individuals and the red bars indicate the HIV-positive individuals. The numbers in/above the bars indicate the number of individuals in that group.

Table 2.3.4 Association between CAPRISA Acute Infection cohort haplotypes and HIV acquisition

Haplotype		%HIV (n/N)	Unadjusted Hazard Ratio (95% CI)	*p*-value
AAT	Yes	13.6% (14/103)	1.37 (0.62 – 3.01)	0.44
	No	10.2% (11/108)	1.00 (reference)	
AGA	Yes	0.0% (0/1)	-	-
	No	11.9% (25/210)	-	
AGT	Yes	12.5% (1/8)	1.07 (0.15 – 7.92)	0.95
	No	11.8% (24/203)	1.00 (reference)	
CAA	Yes	14.3% (1/7)	1.13 (0.15 – 8.33)	0.91
	No	11.8% (24/204)	1.00 (reference)	
CAT	Yes	12.1% (15/124)	1.05 (0.47 – 2.34)	0.90
	No	11.5% (10/87)	1.00 (reference)	
CGA	Yes	9.6% (7/73)	0.71 (0.30 – 1.70)	0.44
	No	13.0% (18/138)	1.00 (reference)	
CGT	Yes	0.0% (0/44)	-	-
	No	15.0% (25/167)	-	

We used a Kaplan-Meier survival curve to determine if -3575 genotype had an association with HIV-infection (see Figure 2.3.2). There seemed to be a trend towards individuals with the -3575AA genotype being more likely to become HIV-infected, however this did not reach significance (p= 0.09).

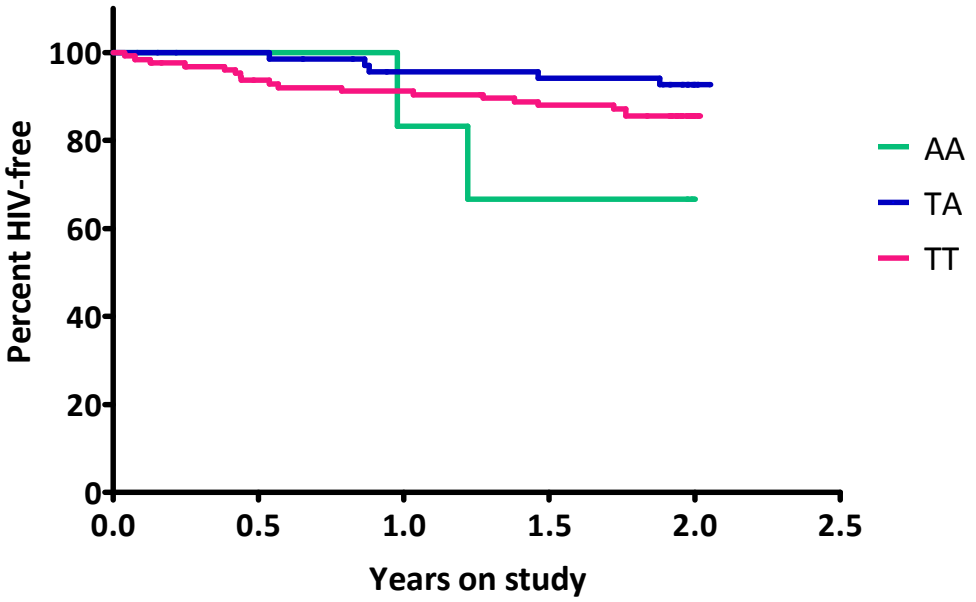

Figure 2.3.2 Kaplan-Meier graph showing the percent of individuals remaining HIV-negative based on *IL-10* -3575 genotype. Data shows that individuals with the -3575AA genotype were more likely to become infected, however this did not reach significance (p= 0.09).

2.3.5 ASSOCIATION OF *IL-10* VARIANT WITH VIRAL LOAD

For this part of the analysis, we analysed the association between viral load and the -3575 genotype for the CAI cohort. For the HAI and SK cohorts, we analysed the association between all three SNPs, i.e. -592, -1082 and -3575, and viral load (see Table 2.3.5).

We included 25 HIV-positive individuals from the CAI cohort in this part of the analysis. We found no significant association between the median viral load at three months and -3575 genotype. We next looked at the association between baseline viral load measurements and -592, -1082 and -3575 genotypes in the HAI and SK cohorts. This sample contained 22 individuals from the HAI cohort, and 300 individuals from the SK cohort. To determine if there was any association between *IL-10* variant and baseline log viral load, we used the Wilcoxon rank sum test. We found no significant association between viral load and any *IL-10* variant for both the HAI and SK cohorts (Table 2.3.5).

Longitudinal data for the CAI cohort was also analysed to see if there was any association with -3575 genotype and viral load over time (see Table 2.3.6). We found no significant association between -3575 genotype and median viral load at any of the three time points measured. We also fitted a linear mixed model to log viral load to measure its association with -3575 genotype over time. Taking into account repeated measures as well as adjusting for viral load, we found no significant association between genotype and viral load (p= 0.83).

Table 2.3.5 Association between *IL-10* variant and baseline median viral load (log copies/ml) in the study cohorts

Cohort	Genotype: -592				Genotype: -1082				Genotype: -3575			
	CC	CA	AA	*p*-value	AA	AG	GG	*p*-value	TT	TA	AA	*p*-value
CAPRISA Acute Infection	-	-	-	-	-	-	-	-	4.6	4.8	4.3	0.60
HPP Acute Infection	5.7	5.8	5.3	0.57	5.2	5.8	5.3	0.24	5.5	5.8	4.8	0.24
Sinikithemba	4.8	4.8	4.8	0.41	4.8	4.8	4.7	0.40	4.6	4.9	4.6	0.23

Table 2.3.6 Association between -3575 genotype and median viral load longitudinally in the CAPRISA Acute Infection cohort

Genotype	3 Months Log VL	9 Months Log VL	12 Months Log VL
	Median (IQR)	Median (IQR)	Median (IQR)
AA	4.3 (4.3 – 6.3)	4.7 (3.5 – 4.9)	4.9 (3.6 – 6.1)
TA	4.8 (4.1 – 5.2)	4.2 (4.0 – 4.7)	4.3 (4.0 – 4.6)
TT	4.6 (3.8 – 4.9)	4.7 (4.3 – 5.2)	4.8 (4.3 – 5.2)
p-value	0.60	0.29	0.23

Based on the three SNPs that were genotyped, i.e. -592, -1082 and -3575, haplotypes were generated and analyses were done to determine if there was an association between viral load and any haplotype within the three cohorts that were included in this study.

We looked at association between *IL-10* haplotype in the CAI and viral load post-infection. First we wanted to determine if there was an association between viral load and time post-infection, specifically at 0-3, and 3-12 months post-infection (see Table 2.3.7). During the first three months of infection, individuals with the AAT haplotype had lower median viral loads (4.5 vs. 4.96 log/copies/ml, p= 0.03). Those individuals with the CGT haplotype had a significantly higher median viral load (5.72 vs. 4.64 log copies/ml, p= 0.04)

We next investigated association between haplotype and viral load during the first year of infection. We analysed viral load over the first 12 months of infection (see Table 2.3.8). A linear model was fitted to each log viral load, adjusting for the repeated measures over time, in order to determine the effect of each haplotype on viral load overall. The viral load models were adjusted for time post-infection and $CD4^+$ T cell count. There was no significant association between any haplotype and mean viral load over the first 12 months post-infection.

Table 2.3.7 The association between CAPRISA Acute Infection cohort haplotype and viral load post-infection

Haplotype		0-3 months		3-12 months	
		Estimated mean (SE)	p-value	Estimated mean (SE)	p-value
AAT	No	4.96 (0.16)	**0.03**	4.39 (0.16)	0.86
	Yes	4.50 (0.14)		4.35 (0.13)	
AGA	No	4.69 (0.11)	-	4.37 (0.10)	-
	Yes	-		-	
AGT	No	4.70 (0.11)	0.80	4.40 (0.11)	0.31
	Yes	4.59 (0.43)		3.98 (0.39)	
CAA	No	4.70 (0.11)	0.96	4.36 (0.10)	0.65
	Yes	4.66 (0.71)		4.68 (0.69)	
CAT	No	4.72 (0.16)	0.80	4.44 (0.15)	0.52
	Yes	4.67 (0.15)		4.30 (0.14)	
CGA	No	4.62 (0.13)	0.31	4.40 (0.13)	0.70
	Yes	4.85 (0.19)		4.31 (0.18)	
CGT	No	4.64 (0.11)	**0.04**	4.34 (0.10)	0.21
	Yes	5.72 (0.49)		4.97 (0.48)	

Table 2.3.8 Association between haplotype and viral load over the first 12 months of infection in the CAPRISA Acute Infection cohort

Haplotype		Log Viral Load	
		Estimated mean (SE)	*p*-value
AAT	No	4.56 (0.15)	0.43
	Yes	4.40 (0.13)	
AGA	No	4.47 (0.10)	-
	Yes	-	
AGT	No	4.50 (0.10)	0.34
	Yes	4.12 (0.39)	
CAA	No	4.47 (0.10)	0.69
	Yes	4.73 (0.67)	
CAT	No	4.54 (0.14)	0.52
	Yes	4.41 (0.14)	
CGA	No	4.47 (0.12)	0.98
	Yes	4.47 (0.17)	
CGT	No	4.44 (0.10)	0.12
	Yes	5.18 (0.47)	

To determine if there was any association between haplotype and log viral load, the Wilcoxon rank sums test was used. Table 2.3.9 shows the association between *IL-10* haplotype and the HAI cohort. Only the CGT haplotype showed a significant impact on viral load, with those 4 individuals having higher viral load measurements than those without the CGT haplotype (6.2 vs. 5.4 log copies/ml, p= 0.02).

Table 2.3.9 Association between HPP Acute Infection cohort haplotypes and log viral load

Haplotype		n	Median Log VL (IQR)	*p*-value
AAT	No	9	5.66 (4.87 - 5.85)	0.66
	Yes	9	5.57 (5.34 - 6.12)	
CAA	No	17	5.66 (5.14 - 6.12)	0.44
	Yes	1	5.00 (5.00 - 5.00)	
CAT	No	10	5.69 (5.14 - 6.12)	0.51
	Yes	8	5.50 (4.85 - 5.96)	
CGA	No	9	5.52 (5.14 - 6.22)	0.72
	Yes	9	5.66 (5.00 - 5.80)	
CGT	No	14	5.43 (4.87 - 5.75)	**0.02**
	Yes	4	6.17 (5.99 - 6.34)	

We next analyzed for association between baseline viral load and the *IL-10* haplotype in the SK cohort (see Table 2.3.10). We found that there was no significant association between any haplotype and baseline viral load in the SK cohort.

Table 2.3.10 Association between *IL-10* haplotypes in the Sinikithemba cohort and baseline viral load

Haplotype		n	Median Log VL (IQR)	*p*-value
AAT	No	178	4.72 (3.95 - 5.27)	0.62
	Yes	246	4.86 (3.97 - 5.26)	
CAA	No	412	4.76 (3.96 - 5.27)	0.43
	Yes	12	4.99 (4.50 - 5.27)	
CAT	No	194	4.79 (3.90 - 5.21)	0.75
	Yes	230	4.71 (4.10 - 5.32)	
CGA	No	259	4.70 (3.95 - 5.27)	0.62
	Yes	165	4.85 (3.99 - 5.26)	
CGT	No	345	4.77 (3.97 - 5.27)	0.87
	Yes	79	4.76 (3.92 - 5.21)	

2.3.6 ASSOCIATION OF *IL-10* VARIANTS WITH $CD4^+$ T CELL COUNT

For this part of the analysis we determined the association between the -3575 genotype and $CD4^+$ T cell count for individuals in the CAI cohort, and the association between all three SNPs, i.e. -592, -1082 and -3575 for individuals in both the HAI and SK cohorts (see Table 2.3.11).

To determine if there was an association between -3575 genotype and $CD4^+$ T cell count for individuals in the CAI cohort, we analyzed median $CD4^+$ T cell count during the first three months post-infection. Here we included 25 HIV-positive individuals from the CAI cohort. The association between $CD4^+$ T cell count and -3575 genotype is shown in Table 2.3.11. We found no significant association between -3575 genotype and $CD4^+$ T cell count. We also analysed *IL-10* genotypes for 22 HIV-positive individuals from the HAI cohort, and 300 HIV-positive individuals from the SK cohort. These baseline $CD4^+$ T cell counts showed no association between any *IL-10* variant.

We analysed $CD4^+$ T cell counts longitudinally for the first 12 months of infection for individuals in the CAI cohort (see Table 2.3.12). We found no significant association between -3575 genotype and $CD4^+$ T cell count during any of the three time points measured. We investigated association between -3575 genotype and median $CD4^+$ T cell count longitudinally over the first 12 months overall. For this part of the analysis we fitted a linear model to $CD4^+$ T cell count to determine its association with -3575 genotype. Taking into account the repeated measurements performed and adjusting for viral load, we found no significant association overall (p= 0.29).

Table 2.3.11 Association between baseline median CD4$^+$ T cell (cells/µl) count and *IL-10* variant in the study cohorts

Cohort	Genotype: -592				Genotype: -1082				Genotype: -3575			
	CC	CA	AA	*p*-value	AA	AG	GG	*p*-value	TT	TA	AA	*p*-value
CAPRISA Acute Infection	-	-	-	-	-	-	-	-	613	499	539	0.41
HPP Acute Infection	417	425	385	0.78	303	492	418	0.28	375	417	554	0.36
Sinikithemba	397	339	341	0.22	341	375	431	0.23	339	368	431	0.21

Table 2.3.12 Association between -3575 genotype and CD4$^+$ T cell count longitudinally in the CAPRISA Acute Infection cohort

Genotype	3 Months CD4	9 Months CD4	12 Months CD4
	Median (IQR)	Median (IQR)	Median (IQR)
AA	613 (255 – 727)	416 (249 – 599)	341 (202 – 479)
TA	499 (374 – 647)	424 (396 – 485)	410 (396 – 470)
TT	539 (445 – 766)	397 (316 – 710)	390 (313 – 642)
p-value	0.41	0.85	0.70

The analysis for the CAI cohort was broken down to compare the association between haplotype and $CD4^+$ T cell count at different phases post-infection, and longitudinally. Table 2.3.13 shows the association between haplotype and $CD4^+$ T cell count at 0-3, and 3-12 months post-infection. The Wilcoxon rank sums test was used. We found no significant association between haplotype and $CD4^+$ T cell count at either 0-3 or 3-12 months post-infection.

The next analysis we did was to determine if there was an association between CAI haplotype and $CD4^+$ T cell count over time. A linear mixed model was fitted to each $CD4^+$ T cell count, while adjusting for multiple measurements over time. $CD4^+$ T cell count models were adjusted for time post infection and viral load. The overall change in the first year of infection is shown in Table 2.3.14. This data shows that in the first 12 months of infection, there was no association between any haplotype and $CD4^+$ T cell count in the CAI cohort.

Table 2.3.13 Association between CAPRISA Acute Infection cohort *IL-10* haplotypes and $CD4^+$ T cell count post infection

Haplotype		0-3 months		3-12 months	
		Estimated mean (SE)	p-value	Estimated mean (SE)	p-value
AAT	No	519.64 (41.45)	0.26	472.0 (40.33)	0.29
	Yes	581.94 (35.46)		527.96 (34.20)	
AGA	No	555.57 (26.86)	-	504.55 (26.09)	-
	Yes	-		-	
AGT	No	543.41 (27.18)	0.08	493.88 (26.65)	0.12
	Yes	726.85 (101.85)		352.47 (99.33)	
CAA	No	554.68 (27.48)	0.83	500.83 (26.42)	0.35
	Yes	593.09 (180.55)		666.79 (174.49)	
CAT	No	549.76 (38.83)	0.83	492.49 (37.58)	0.65
	Yes	561.10 (38.00)		51.18 (36.96)	
CGA	No	585.02 (32.42)	0.12	543.81 (31.32)	0.1
	Yes	495.95 (46.18)		444.76 (44.08)	
CGT	No	559.09 (27.71)	0.55	508.96 (26.84)	0.43
	Yes	479.54 (129.15)		408.70 (125.06)	

Table 2.3.14 Association between CAPRISA Acute Infection cohort haplotype and CD4$^+$ T cell count over the first 12 months of infection

Haplotype		CD4 count	
		Estimated mean (SE)	*p*-value
AAT	No	486.13 (39.25)	0.26
	Yes	543.88 (33.42)	
AGA	No	519.60 (25.50)	-
	Yes	-	
AGT	No	508.60 (25.92)	0.10
	Yes	673.24 (96.79)	
CAA	No	516.53 (25.89)	0.43
	Yes	654.00 (171.21)	
CAT	No	508.23 (36.76)	0.67
	Yes	530.53 (36.05)	
CGA	No	549.93 (30.55)	0.09
	Yes	459.21 (43.12)	
CGT	No	523.28 (26.26)	0.51
	Yes	440.04 (122.11)	

We next investigated the association between *IL-10* haplotype and $CD4^+$ T cell count in the HAI and SK study cohorts. Table 2.3.15 shows the association between HAI haplotypes and $CD4^+$ T cell count at baseline. The Wilcoxon rank sums test was used to determine if there was an association between any haplotype and $CD4^+$ T cell count. We found no significant association between any of the haplotypes and $CD4^+$ T cell count at baseline.

Table 2.3.15 Association between HPP Acute Infection cohort haplotypes and baseline $CD4^+$ T cell count

Haplotype		n	Median CD4 (IQR)	*p*-value
AAT	No	12	417 (276 - 526)	0.67
	Yes	10	401 (304 - 626)	
CAA	No	21	411 (304 - 514)	0.16
	Yes	1	676 (676 - 676)	
CAT	No	12	415 (339 - 570)	0.34
	Yes	10	363 (242 - 559)	
CGA	No	10	375 (245 - 626)	0.45
	Yes	12	425 (314 - 537)	
CGT	No	16	414 (275 - 537)	0.63
	Yes	6	404 (318 - 626)	

We next studied the association between the SK cohort haplotypes and baseline $CD4^+$ T cell count (see Table 2.3.16). The Wilcoxon rank sums test was used here and we found no significant association between any *IL-10* haplotype and baseline $CD4^+$ T cell count.

Table 2.3.16 Association between Sinikithemba cohort haplotypes and baseline $CD4^+$ T cell count

Haplotype		n	Median CD4 (IQR)	*p*-value
AAT	No	177	376 (251 - 513)	0.32
	Yes	247	339 (231 - 492)	
CAA	No	412	351 (239 - 506)	0.75
	Yes	12	409 (175 - 593)	
CAT	No	195	357 (230 - 516)	0.73
	Yes	229	352 (254 - 481)	
CGA	No	260	339 (238 - 468)	0.15
	Yes	164	380 (242 - 517)	
CGT	No	345	347 (241 - 505)	0.63
	Yes	79	373 (227 - 513)	

2.3.7 ASSOCIATION OF GENOTYPE WITH $CD4^+$ T CELL DECLINE IN CHRONIC HIV-1 INFECTION

As we wanted to determine the effect of IL-10 genetic variants on CD4 decline during chronic infection we focused on the SK cohort. We investigated at the association between the -592 and -1082 genotypes and $CD4^+$ T cell decline. We included 300 individuals with follow-up data. As individuals in the SK cohort may not be at the same stage of chronic infection, we therefore stratified the data according to viral load and $CD4^+$ T cell count to reduce any bias which may be introduced due to participants being in different phases of infection.

Figure 2.3.3 shows the rate of CD4 decline during the first 24 months of follow-up. CD4 decline was stratified according to $CD4^+$ T cell count and viral load as follows: CD4>350, VL>100 000; CD4>350, VL$\leq$100,000; CD4$\leq$350, VL>100,000; CD4$\leq$350, VL$\leq$100,000. Across all strata we found that the -592AA genotype (indicated by pink bars), had an attenuated loss of $CD4^+$ T cells during the first 24 months of follow-up, which was significant (p= 0.05). Similar analysis was performed for the -1082 genotype (see Figure 2.3.4). Although in all strata the -1082AA genotype had an attenuated loss of $CD4^+$ T cells, this did not reach significance (p= 0.15).

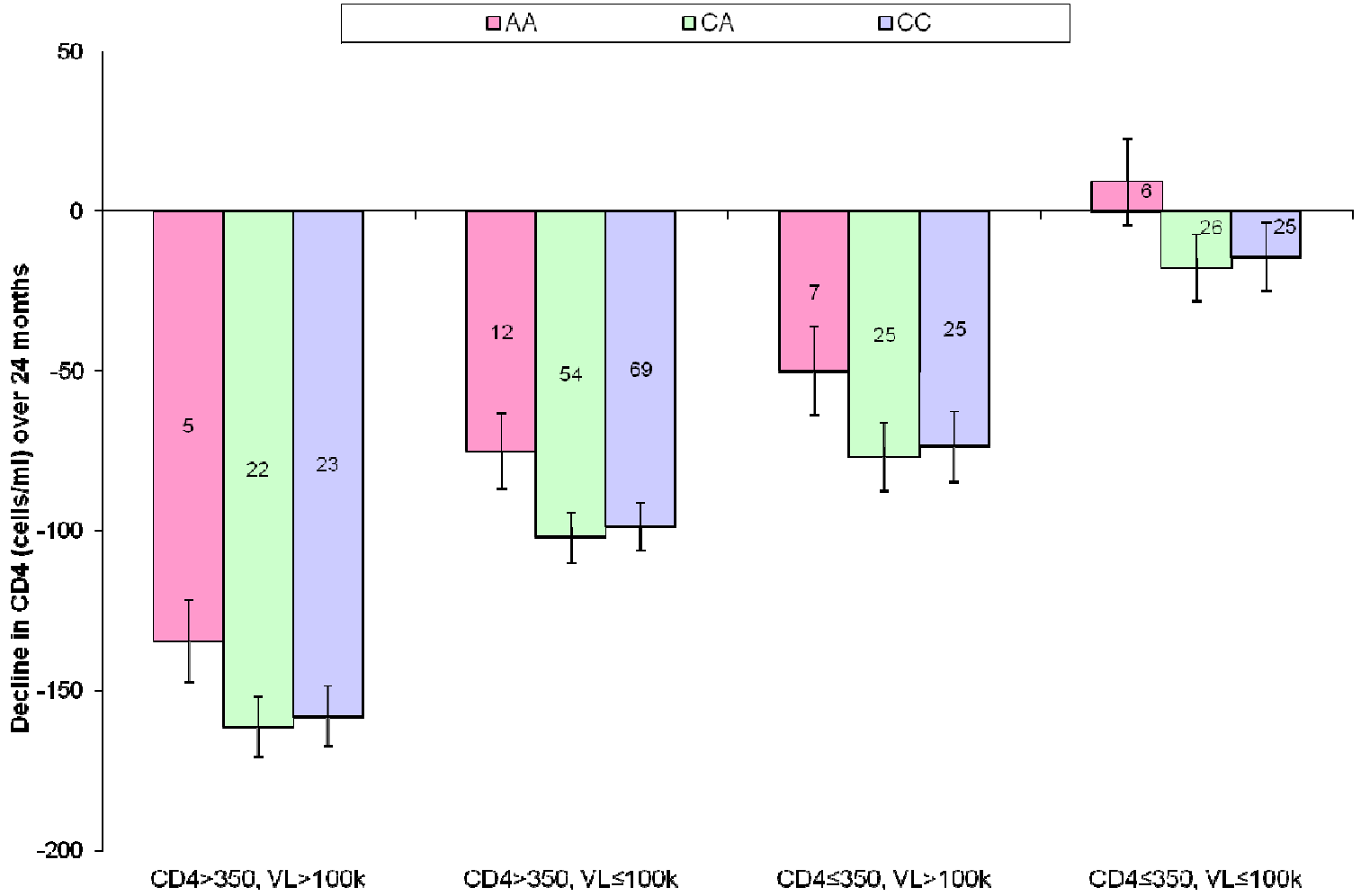

Figure 2.3.3 Association between -592 genotype and CD4 decline in the Sinikithemba cohort. The -592AA genotype (pink bars) had an attenuated loss of $CD4^+$ T cells over all strata, which was significant (p= 0.05).

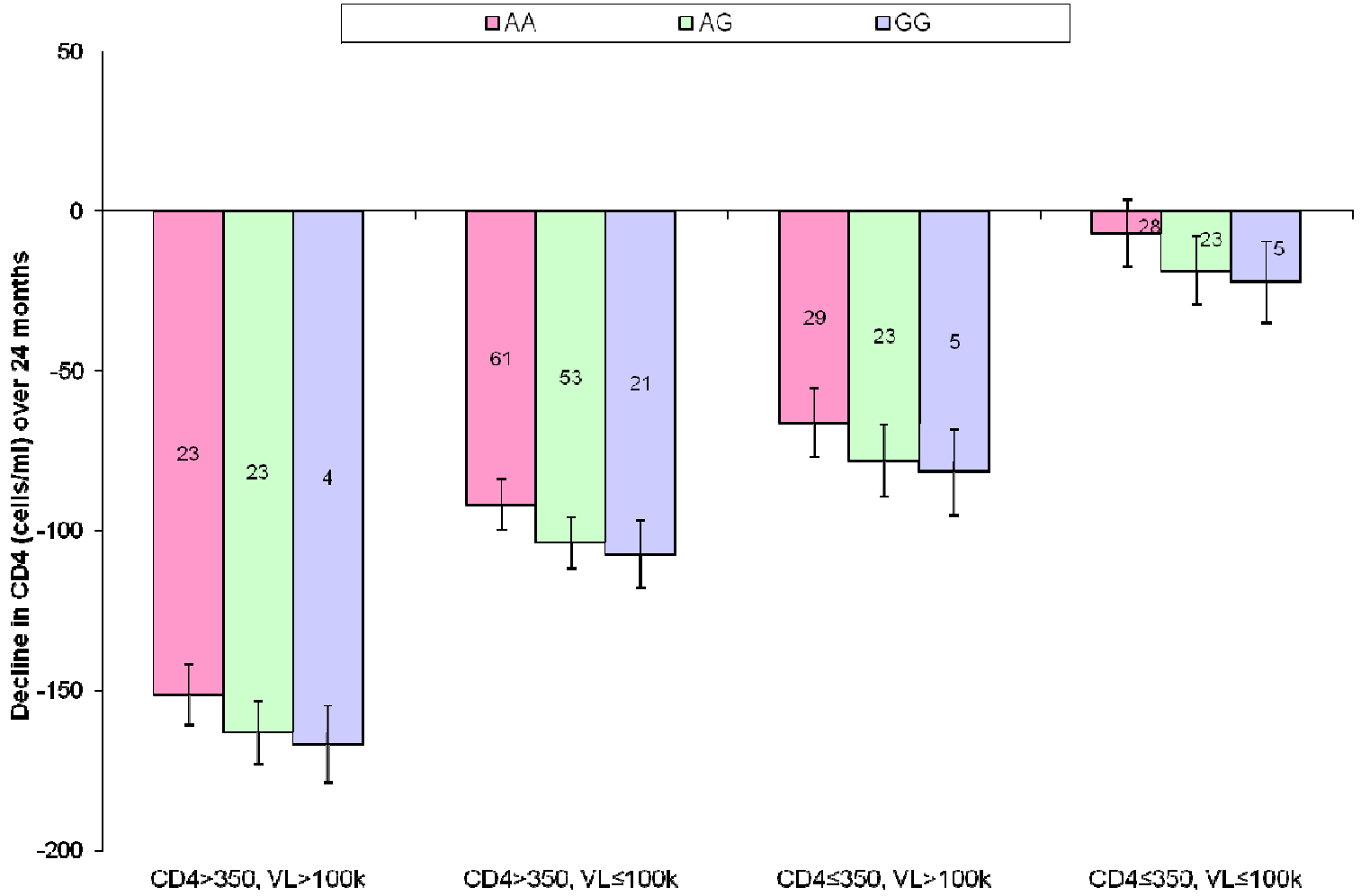

Figure 2.3.4 The association between -1082 genotype and CD4 decline in the Sinikithemba cohort. The -1082AA genotype (pink bars) seemed to trend towards an attenuated loss of $CD4^+$ T cells over all strata, however this did not reach significance (p= 0.15).

2.4 DISCUSSION

We wanted to determine if previously identified *IL-10* promoter polymorphisms were present in our predominantly black African study population. We therefore genotyped a total of 685 individuals from three different cohorts of HIV-1 infection, of which 498 had either acquired HIV-1 infection or were HIV-positive upon enrollment to the chronic cohort. The three cohorts included in this study were the CAPRISA Acute Infection (CAI) cohort, the HPP Acute Infection (HAI) cohort, and the HPP Sinikithemba (SK) cohort of Chronic Infection. These three cohorts allowed us to study both HIV-negative individuals as well as HIV-positive individuals at different stages of infection.

We investigated the association of three *IL-10* promoter polymorphisms, i.e. -592, -1082 and -3575, with biomarkers of HIV-1 infection, such as viral load, $CD4^+$ T cell count and the rate of CD4 decline. All cohorts and subgroups within cohorts were found to be in Hardy-Weinberg equilibrium, using the Chi-Square test. This means that the observed allele frequencies were not significantly different from the expected allele frequencies. This confirms that the study population and subpopulations used in analyses did not deviate from Hardy-Weinberg equilibrium, and that there was no underlying forces driving these allele frequencies, such as non-random mating, mutations, selection, random genetic drift, gene flow and meiotic drive.

We previously studied the CAI cohort, focusing on the -592 and the -1082 polymorphisms and biomarkers of HIV-1 infection (Naicker et al., 2009). Here we found that individuals with *IL-*

10 polymorphisms associated with high-IL-10 production were less likely to become infected, and once infected these individuals had significantly higher median viral loads during the acute phase of infection. However, as infection progressed the association between high-IL-10-producing genotypes and high viral load seemed to reverse or the association was lost during the later stages of infection. Therefore it seems that the role of IL-10 is dependent on the stage of infection.

In this study we characterised the -3575 genotype in 212 individuals from the CAI cohort, 25 of which had seroconverted during the follow-up period. Using the Kaplan-Meier survival curve, we looked at the association between the -3575 genotype and time to infection. There was no significant association between the -3575 genotype and susceptibility to HIV infection. However, we found that there seemed to be a trend individuals with the -3575AA genotype being more susceptible to HIV infection, as compared to individuals with the other 3575 genotypes, however this did not reach statistical significance (p= 0.09). Interestingly, we found that the CGT haplotype had an association with HIV acquisition. We found that all individuals with the CGT haplotype were HIV-negative (Fisher's exact test, p= 0.003). The three alleles that make up this haplotype are all associated with high IL-10 production. Therefore, individuals with this haplotype were more likely to have increased IL-10 production, which may have protecting them against HIV infection.

To determine if there was an association between viral load and $CD4^+$ T cell count and -3575 genotype in the CAI cohort, we looked at longitudinal measurements at 3, 9 and 12 months post-infection. However, we did not find any association between any -3575 genotype and either viral load or $CD4^+$ T cell count at any time point.

We analyzed the *IL-10* haplotype data for association between haplotypes and viral load or $CD4^+$ T cell count longitudinally. We found no association between any *IL-10* haplotypes and cross-sectional measurements of $CD4^+$ T cell counts at 0-3, and 3-12 months post-infection. Also, there was no association between the *IL-10* haplotype and the first 12 months of infection. However, when we broke down the first 12 months into 0-3 and 3-12 months post-infection, we found that the AAT and CGT haplotypes associated significantly with viral load. Individuals with the AAT haplotype and those without the CGT haplotype had lower viral loads during the first 3 months of infection. The same time point or infection phase breakdown approach was used for $CD4^+$ T cell count. We found no significant association between $CD4^+$ T cell count, and any haplotype during the first 12 months of follow-up.

We next studied the HAI and the SK Chronic Infection cohorts. Here we characterised three *IL-10* promoter polymorphisms, i.e. -592, -1082 and -3575. At the time of assessment we included 22 acutely infected individuals from the HAI cohort, and 451 individuals in the chronic phase of infection from the SK cohort. We wanted to determine if there was an association between biomarkers of HIV-1 infection and *IL-10* variants. To do this we first determined if there was an association between *IL-10* variants and either viral load or $CD4^+$ T cell count at the baseline (entry) time point. There was no significant association between any *IL-10* haplotype and either viral load or $CD4^+$ T cell count at the baseline time point. In this cross-sectional analysis, we did not find any association between any *IL-10* variant and either viral load or $CD4^+$ T cell count.

We then focused on the SK cohort, investigating the rate of CD4 decline in 300 individuals with follow-up data for the first 24 months. We wanted to determine if there was an

association between any *IL-10* variant and the rate of CD4 decline. Here we focused on the two proximal IL-10 promoter polymorphisms, i.e. -592 and -1082. The data was stratified according to $CD4^+$ T cell count and viral load. Here we found that individuals with *IL-10* genotypes that have been previously found to be associated with low-IL-10-production, had an attenuated loss of $CD4^+$ T cells during the first 24 months of follow-up, this did not reach significance for the -1082 genotype, but was significant for the -592 genotype (p= 0.15 and 0.05, respectively).

The genotype data is consistent with the findings of Shin *et al* (Shin et al., 2000), as they found that high-IL-10-producing genotypes were associated with protection against infection, but an acceleration to AIDS, particularly during the late stages of infection. We hypothesise that during the early stages of infection, higher IL-10 levels (and by extension, high-IL-10-producing genotypes) can dampen the antiviral adaptive and innate effector mechanisms, resulting in poor control of viral replication (Alter et al., 2010, Herbein and Varin, 2010, Martinic and von Herrath, 2008). However, the beneficial effects of IL-10 are more pronounced during the later stages of infection by its anti-inflammatory effects and the direct inhibition of HIV-1 replication within macrophages (Ancuta et al., 2001, Bento et al., 2009, Wang and Rice, 2006).

We did not see any association between the -3575 genotype and either viral load or $CD4^+$ T cell count for the CAI cohort. As the sample size of the HIV-positive individuals included was small, this may explain why we were not able to see the effect of the -3575 in this small sample size. Similarly, the small sample size of the HAI cohort, may have limited our power to detect any effects of *IL-10* variants on viral load and $CD4^+$ T cell count. As the AAT and

CGT haplotypes associated with viral load, we would need to further investigate the phenotypes of these haplotypes in this cohort, to determine the effect of IL-10 on viral load.

There are some limitations to the SK cohort, which may account for no significance in the association between *IL-10* variants and viral load or $CD4^+$ T cell counts in this cross-sectional analysis. As the time since HIV-1 infection is unknown for study participants in the SK cohort, a survivor bias may have been introduced. Also, as we do not know time of infection for individuals in the SK cohort, it is possible that we may be analysing individuals at different phases of infection altogether. In order to minimize these limitations, the study population was stratified according to baseline viral loads and $CD4^+$ T cell counts.

In conclusion, this part of the study emphasizes the complex role of *IL-10* genetic variants in HIV-1 susceptibility and pathogenesis. In cross-sectional analysis we found that *IL-10* genetic variants did not associate with viral load and $CD4^+$ T cell counts. However, we did find that there was a trend that *IL-10* variants associated with low-IL-10-production, showed an attenuated loss of $CD4^+$ T cells during the 24 months of follow-up during chronic infection. This may be due to its anti-inflammatory effects and the role IL-10 plays in the direct inhibition of HIV replication within macrophages, which have a more pronounced effect in the late stages of infection when the $CD4^+$ T cells have become depleted. However, mechanistic studies are needed to address this hypothesis.

Chapter 3

Association between *IL-10* variants, cytokine expression and immune responses

Chapter 3: Association between *IL-10* variants, cytokine expression and immune responses

INTRODUCTION

Interleukin-10 is a potent, anti-inflammatory cytokine that has been shown to play a role in immunoregulation. This powerful cytokine is produced by Th2 cells, and has been shown to down-regulate the expression of MHC class I and class II antigens, and numerous other proinflammatory Th1 cytokines.

The expression of cytokines is under genetic control (Hutchinson et al., 1998). Polymorphisms found within the promoter (regulatory) region of these genes can affect gene expression and disease pathogenesis. The promoter region of *IL-10* has polymorphisms in both the proximal and distal regions of the promoter. The proximal region has three well studied single nucleotide polymorphisms (SNPs), found upstream from the transcription start site, at positions -592, -819 and -1082. The -592 and -819 SNPs are in complete linkage disequilibrium, so the mutations will always be inherited together.

Polymorphisms at these positions have been shown to affect IL-10 production. An A to G transition at position -1082 has been shown to be associated with an increase in IL-10 production *in vitro* (Turner et al., 1997). Previous studies report that the wild-type -1082A allele and the polymorphism -592A alleles are associated with low IL-10 production (Hutchinson et al., 1998, Turner et al., 1997). Previous studies have also shown that haplotypes, resulting from the combination of the three proximal SNPs have varying affects on IL-10 production (Eskdale et al., 1998). Based on the -1082, -819 and -592 combination of alleles, the GCC/GCC haplotypes is associated with high IL-10 production, the GCC/ATA or

GCC/ACC haplotypes with intermediate IL-10 production and the ATA/ATA, ATA/ACC or ACC/ACC haplotypes with low IL-10 production (Edwards-Smith et al., 1999).

Also, the -1082G polymorphism is recessive, which means that to see the effect of the -1082G being associated with high IL-10 production the genotype has to be homozygous for the polymorphism, i.e. -1082GG. Therefore both the -1082AA and -1082AG are associated with low IL-10 production. The -592A polymorphism is dominant. So, if an individual is a carrier of the mutation, the effect of the -592A allele (associated with low-IL-10-production) can be seen, i.e. either -592AA or -592CA (Shin et al., 2000).

The -3575 SNP is found in the distal region of the *IL-10* promoter region. The polymorphism at this region is a T to A transversion. This -3575A polymorphism has previously been shown to be associated with low IL-10 production (Gibson et al., 2001). Gibson *et al* studied the effect of *IL-10* polymorphisms on IL-10 production, and its association with Systemic Lupus Erythematosus**.** In this study they showed that when characterising the -3575 in the healthy control group, they found that the -3575T allele was at a much higher frequency in the African American group than the Dutch Caucasian group. Here they found no significant difference between the distribution of the -3575 genotype in the control individuals and individuals with Systemic Lupus Erythematosus (SLE).

Infection with HIV stimulates strong immune responses by cytotoxic T lymphocytes (CTLs). During the acute phase of HIV infection, the rise of viral load results in a CTL response (McMichael and Rowland-Jones, 2001). When the CTL response reaches a peak, the viral load levels drop, this results in an inverse relationship between CTL responses and viral load. The

CTL response during HIV infection is also influenced by the individual's human leukocyte antigen (HLA) type (Carrington et al., 1999, Kaslow et al., 1996). Different HLA molecules present different virus peptides for recognition by the CTLs, the HLA type of an individual is important because different HLA types present different HLA peptides, which therefore determine the specificity of the immune response.

Mechanistic studies of the lymphocytic choriomeningitis virus (LCMV) in the mouse model showed that IL-10 gene knock-out or signaling blockade enhanced T-cell immune responses, which resulted in rapid viral elimination and the development of antiviral memory T-cell response (Brooks et al., 2006, Ejrnaes et al., 2006).

As our data showed that there was an attenuated loss of CD4 cells in individuals with *IL-10* variants associated with low IL-10 production, the aim of this part of the study was to determine if *IL-10* variants did in fact impact the expression of IL-10 in an HIV setting, as the role of *IL-10* variants on IL-10 expression during HIV-1 infection had not previously been studied. We also wanted to determine if the levels of IL-10 had any effect on biomarkers of HIV-1 infection or the level of expression of other pro-inflammatory cytokines, such as IFN-γ, IL-2, IL-6 and TNF-α. We next wanted to determine if *IL-10* variants or levels of IL-10 influenced the breadth (number of HIV peptides targeted by CTL) or magnitude (number of IFN-γ producing cells per million PBMCs) of HIV-1 specific immune responses *in vivo,* as measured by IFN-γ ELISPOT. This data would allow us to understand the role of *IL-10* genetic variants and the expression of IL-10 in an HIV setting, the influence on other pro-inflammatory cytokines and the effect on the breadth and magnitude of CTL response.

3.2 MATERIALS AND METHODS

3.2.1 STUDY POPULATION

We measured the cytokine profile in a subset of 112 individuals chronically infected with HIV-1 subtype C. These were individuals from the HPP Sinikithemba chronic infection cohort. Cytokine measurements were done using plasma samples. These plasma samples are routinely separated from whole blood by centrifugation and stored at -80^{o}C until further use.

HIV-1 specific CTL responses were measured in a subset of 409 individuals from the Sinikithemba cohort and 22 individuals from the HPP Acute Infection cohort.

3.2.2 CYTOKINE PROFILING USING LUMINEX METHODOLOGY

Luminex methodology was used to simultaneously measure the levels of both pro- and anti-inflammatory cytokines in individuals chronically infected with HIV-1 subtype C. An overview of the technique is shown in Figure 3.2.1. With luminex methodology microspheres that are 5.6 microns in size are dyed to create 100 distinct colours through internal colouring of the bead. Two fluorescent dyes are used, therefore the precise concentrations of these fluorescent dyes creates 100 distinctly coloured bead sets. These beads are coated with a specific capture antibody, therefore this methodology has the potential to analyse 100 different analytes simultaneously. When the sample is added to the microspheres the analyte is captured. A biotinylated detection antibody is introduced and the sample is then incubated with Streptavidin-PE conjugate, which is the reporter molecule, which completes the reaction

on the surface of each microsphere. When the microspheres are passed through a laser which excites the fluorescent dyes within the microsphere, the red laser classifies the bead classification, i.e. what is being analysed, and the green laser classifies the assay result, i.e. the concentration of the analyte. The results are therefore quantified based on the fluorescent reporter signals.

Plasma IFN-γ, IL-2, IL-6, IL-10 and TNF-α concentration was determined by Luminex methodology, using the Millipore Milliplex^{TM} MAP High Sensitivity Human Cytokine Kit. The assay was followed as per manufacturer's protocol as shown in Figure 3.2.2. Plasma samples were centrifuged at 1,500 rpm for 5 minutes at room temperature, prior to assay setup, to remove particulates which may clog the Microtiter Filter Plate wells. Standards and quality controls are included in each kit. The standards were serially diluted to 1:5 starting with 2000 pg/ml; 400 pg/ml; 80 pg/ml; 16 pg/ml; 3.2 pg/ml; 0.64 pg/ml; 0.13 pg/ml; and 0 (assay buffer alone). These standards were used as a reference for the quantification of the analytes. Briefly wash buffer was added to the microtiter filter plate and placed on a shaker for 10 minutes to wet the well membrane. The detection beads (microspheres) were added to the wells and the supernatant was vacuumed, leaving the beads in the well. The standards, assay buffer, Matrix and samples were added and left to incubate overnight at 4^{o}C. This allowed for the antibody to capture the analytes. After 16-18 hours the samples were vacuumed again, and the detection antibody was added. After an hour of incubation at room temperature, the Streptavidin-PE conjugate was added, which acted as the reporter dye. After incubation at room temperature for 30 minutes, samples were vacuumed and washed twice. Sheath fluid was added and then

samples were quantified on the Luminex 200 (Invitrogen, Life Technologies, Grand Island, NY, USA).

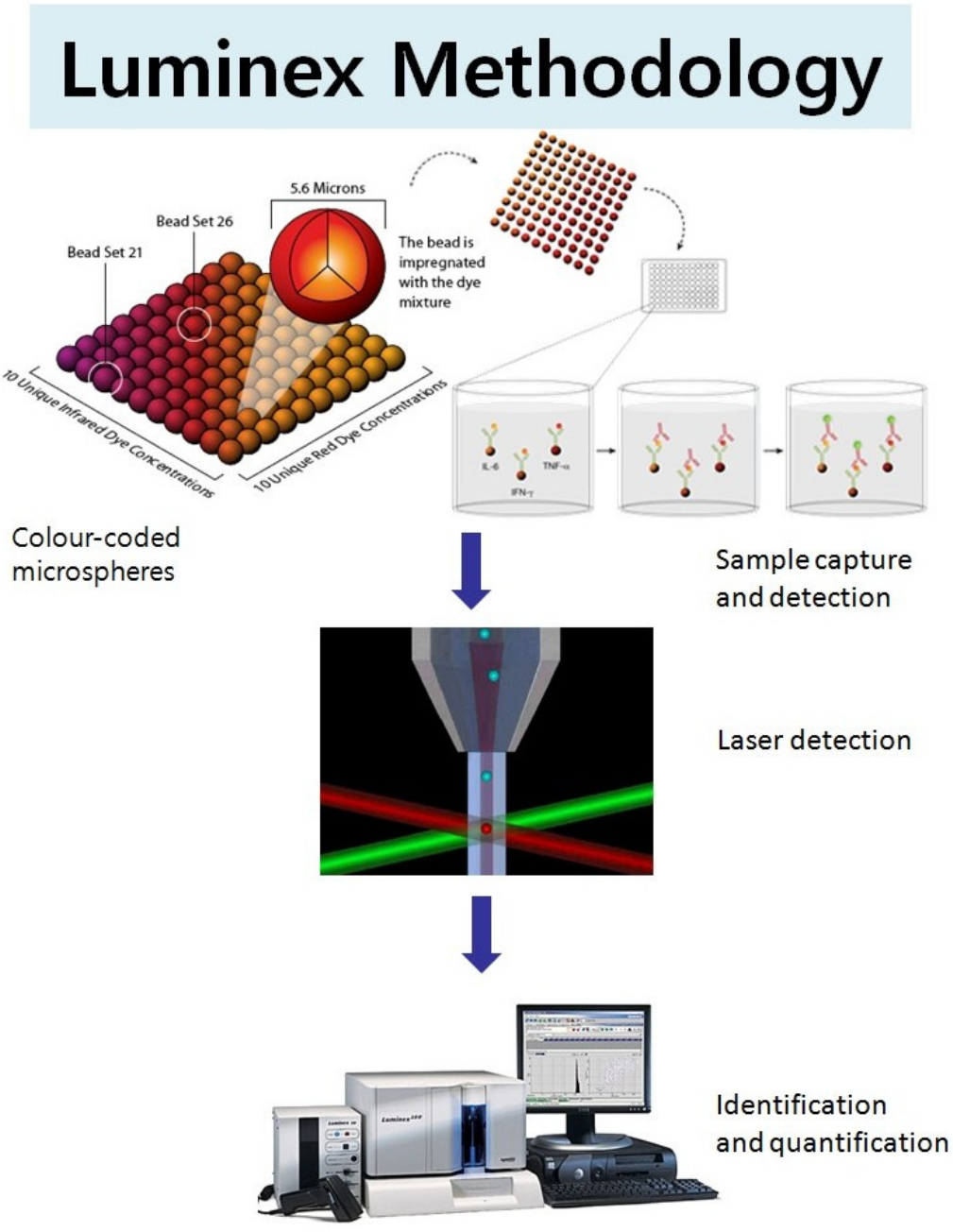

Figure 3.2.1 Overview of Luminex Methodology (images taken from (Bonetta, Invitrogen, Panomics)). Beads are coloured with precise concentrations of two fluorescent dyes, which are coated with specific capture antibodies. When the analyte is added it is captured by the

antibody, a biotinylated detection antibody is added then the samples are incubated with Streptavidin-PE conjugate, which is the reporter molecule. Samples are passed through a laser which excites the fluorescent dyes, and identification and quantification is reported.

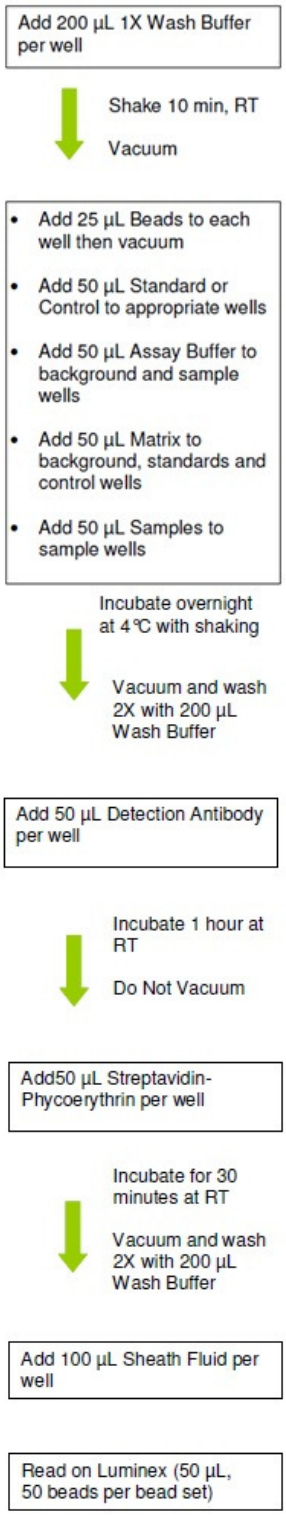

Figure 3.2.2 Overview of Luminex assay (Millipore).

3.2.3 CYTOTOXIC T LYMPHOCYTE RESPONSES

CTLs are measured routinely in the lab at scheduled visits for both the HPP Acute Infection and the HPP Sinikithemba Chronic Infection cohorts. The number and magnitude of HIV peptides targeted by CTL were measured at baseline by interferon-γ (IFN- γ) enzyme-linked immunosorbent (ELISPOT) assay using a panel of 410 overlapping peptides spanning the HIV-1C proteome (Kiepiela et al., 2004, Radebe et al., 2011).

3.2.4 STATISTICAL ANALYSIS

Different methods of statistical analysis were used to determine if there was any association between *IL-10* variants and pro- and anti-inflammatory cytokine production, as well as the association between *IL-10* variants and the breadth and magnitude of immune responses. To determine if there was an association between a specific *IL-10* variant and plasma IL-10 concentration, the Kruskal-Wallis test was used. To determine if there was association between *IL-10* haplotype, cytokine production and the breadth and magnitude of immune responses, we used the Wilcoxon rank sum test. The Pearson's correlation test was used to determine the association between IL-10 concentration and other plasma cytokines. To determine if there was an association between *IL-10* variants and magnitude or breadth of immune responses, we used the Kruskal-Wallis test.

3.3 RESULTS

3.3.1 THE ASSOCIATION BETWEEN *IL-10* VARIANTS AND IL-10 EXPRESSION

To investigate if *IL-10* genetic variants had an effect on IL-10 production in an African setting of chronic HIV-1C infection, we focused on 112 individuals from the HPP Sinikithemba cohort, analysing the association between the -592 and -1082 SNPs with IL-10 production.

We first examined the -592 genotype. We measured the levels of IL-10 expression in plasma from the baseline (entry) time point. The -592 genotypes were grouped according to the dominance pattern of the polymorphism (see Figure 3.3.1). A study by Shin *et al.* (2000) showed that the -592A allele is dominant, therefore all carriers of the -592A allele were grouped together, i.e. -592CA (carriers for the dominant polymorphism) or -592AA (individuals homozygous for the polymorphism). Previous studies show that the -592CC genotype is associated with high IL-10 production (Hutchinson et al., 1998, Turner et al., 1997). In this cross-sectional analysis of IL-10 production, although the -592CC group had higher median level of plasma IL-10, this was not statistically significant (p= 0.22).

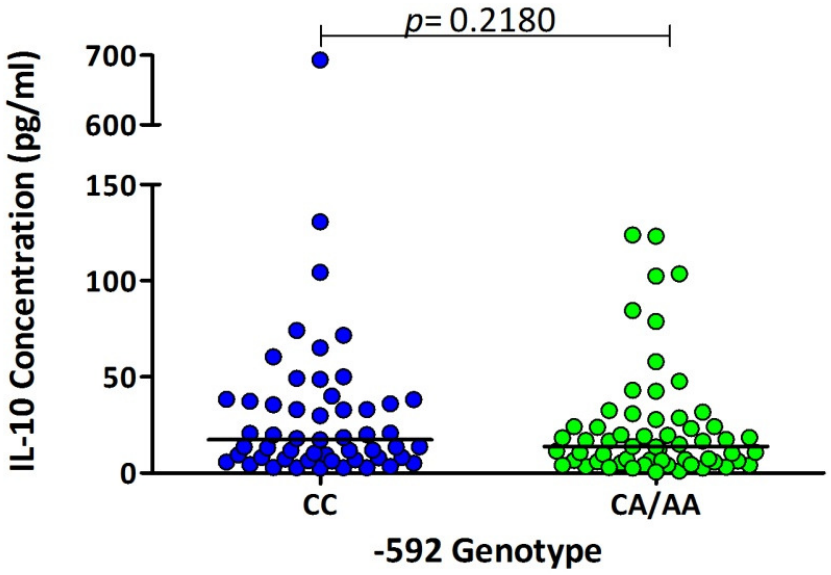

Figure 3.3.1 Association between -592 genotype and IL-10 expression. Although the -592CC group had a higher median expression of IL-10, this was not statistically significant.

We then went on to investigate the association between the -1082 genotype and IL-10 expression for 112 individuals from the HPP Sinikithemba Chronic Infection cohort (see Figure 3.3.2). Based on previous studies, the -1082G polymorphism is shown to be recessive, therefore in order for the high-IL-10-producing effect of the -1082G allele to be seen, the individual would have to be homozygous for the polymorphism. As the -1082G polymorphism is recessive, we grouped carriers of the wild-type -1082A allele together, i.e. -1082AA (homozygous for the wild-type allele) and -1082AG (carrier of the wild-type allele). Here we found that the -1082GG group had a significantly higher median level of IL-10 expression as compared to the combined -1082AA/-1082AG group (p= 0.0006).

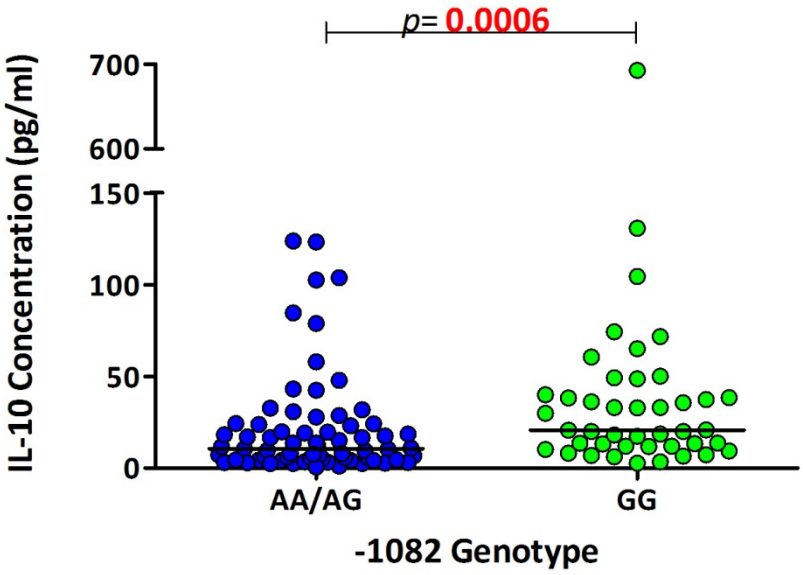

Figure 3.3.2 Association between -1082 genotype and IL-10 expression. The -1082GG group had a significantly higher median level of IL-10 expression as compared to the combined -1082AA/-1082AG group.

We next wanted to determine if *IL-10* haplotypes has an association with cytokine expression in the Sinikithemba Chronic Infection cohort. We measured the expression of IL-10, IL-2, IL-6, IFN-γ and TNF-α. To determine if there was an association between *IL-10* extended haplotype and cytokine expression, we used the Wilcoxon rank sums test. However, as some haplotypes were present in only very small numbers in this subgroup of the SK cohort, groups with less than 5 individuals were excluded from the analysis.

Table 3.3.1 shows the association between IL-10 haplotype and IL-10 expression. We found a significant association between the CAT haplotype and IL-10 expression. Individuals with the CAT haplotype had significantly lower IL-10 expression than individuals without the CAT haplotype (median 5.81 vs. 18.25pg/ml, $p < 0.0001$).

Table 3.3.1 Association between *IL-10* haplotype and IL-10 expression in the Sinikithemba cohort of chronic infection

Haplotype		n	Median IL-10 (IQR)	*p*-value
AAT	No	56	17.66 (8.01 - 38.28)	0.21
	Yes	68	13.73 (6.42 - 27.65)	
CAA	No	123	15.04 (6.75 - 33.02)	-
	Yes	1	9.72	
CAT	No	105	18.25 (8.07 - 37.31)	**<.0001**
	Yes	19	5.81 (3.16 - 9.52)	
CGA	No	73	12.68 (6.25 - 24.23)	0.06
	Yes	51	18.55 (8.11 - 40.02)	
CGT	No	87	15.04 (6.58 - 32.61)	0.8
	Yes	37	13.76 (7.52 - 33.02)	

The association between *IL-10* haplotype and IL-2 expression is shown in Table 3.3.2. Using the Wilcoxon rank sums test, we found that individuals with the CAT haplotype had significantly lower IL-2 expression as compared to those without the CAT haplotype (median 0.29 vs. 0.43 pg/ml, p= 0.03).

Table 3.3.2 The association between *IL-10* haplotype and the expression of IL-2 in the Sinikithemba cohort of chronic infection

Haplotype		n	Median IL-2 (IQR)	*p*-value
AAT	No	56	0.46 (0.22 - 1.76)	0.13
	Yes	65	0.29 (0.18 - 0.90)	
CAA	No	120	0.38 (0.20 - 1.30)	-
	Yes	1	0.18	
CAT	No	102	0.43 (0.19 - 1.50)	**0.03**
	Yes	19	0.29 (0.11 - 0.37)	
CGA	No	70	0.32 (0.21 - 0.91)	0.28
	Yes	51	0.48 (0.16 - 1.82)	
CGT	No	85	0.33 (0.18 - 1.23)	0.72
	Yes	36	0.47 (0.23 - 1.30)	

Table 3.3.3 shows the association between IL-6 expression and *IL-10* haplotype. The CAT haplotype had a significant association between *IL-10* haplotype and IL-6 expression. Individuals with the CAT haplotype had significantly lower IL-6 expression as compared to those without the CAT haplotype (median 0.3 vs. 2.04pg/ml, $p < 0.0001$).

Table 3.3.3 The association between *IL-10* haplotype and IL-6 expression in the Sinikithemba cohort of chronic infection

Haplotype		n	Median IL-6 (IQR)	*p*-value
AAT	No	55	1.70 (0.60 - 4.16)	0.22
	Yes	65	0.99 (0.40 - 3.85)	
CAA	No	119	1.53 (0.47 - 4.16)	0.69
	Yes	1	0.81	
CAT	No	101	2.04 (0.68 - 5.21)	**<.0001**
	Yes	19	0.30 (0.19 - 0.66)	
CGA	No	70	0.96 (0.32 - 3.85)	0.08
	Yes	50	1.72 (0.68 - 4.16)	
CGT	No	84	1.15 (0.38 - 3.91)	0.16
	Yes	36	2.16 (0.68 - 4.63)	

Table 3.3.4 shows the association between IFN-γ expression and *IL-10* haplotype. There was a significant association between IFN-γ expression and *IL-10* haplotype. Individuals with the CAT haplotype had significantly lower IFN-γ expression as compared to those without the CAT haplotype (Median 1.09 vs. 2.81pg/ml, $p= 0.01$).

Table 3.3.4 Association between *IL-10* haplotype and IFN-γ expression in the Sinikithemba cohort of chronic infection

Haplotype		n	Median IFN-γ (IQR)	*p*-value
AAT	No	52	2.81 (0.69 - 10.51)	0.25
	Yes	61	1.69 (0.79 - 5.18)	
CAA	No	112	2.36 (0.74 - 7.59)	-
	Yes	1	0.3	
CAT	No	94	2.81 (0.88 - 10.50)	**0.01**
	Yes	19	1.09 (0.40 - 1.94)	
CGA	No	67	1.79 (0.79 - 5.18)	0.31
	Yes	46	2.93 (0.66 - 12.67)	
CGT	No	78	2.07 (0.66 - 7.62)	0.6
	Yes	35	2.40 (0.88 - 7.00)	

Table 3.3.5 shows the association between the *IL-10* haplotype and TNF-α expression. We found a significant association between the *IL-10* haplotype and TNF-α expression. Individuals with the CAT haplotype had a significantly lower expression of TNF-α as compared to those without (median 3.91 vs. 8.63pg/ml, $p = 0.002$).

Table 3.3.5 Association between *IL-10* haplotype and TNF-α expression in the Sinikithemba cohort of chronic infection

Haplotype		n	Median TNF-α (IQR)	*p*-value
AAT	No	56	8.39 (4.88 - 14.96)	0.6935
	Yes	68	8.39 (4.91 - 14.63)	
CAA	No	123	8.35 (4.84 - 14.82)	-
	Yes	1	14.74	
CAT	No	105	8.63 (5.90 - 14.84)	**0.0024**
	Yes	19	3.91 (2.33 - 9.32)	
CGA	No	73	8.28 (4.65 - 14.53)	0.3607
	Yes	51	8.54 (5.56 - 15.10)	
CGT	No	87	8.63 (4.70 - 14.84)	0.9174
	Yes	37	8.08 (4.92 - 14.72)	

3.3.2 ASSOCIATION BETWEEN IL-10 LEVELS AND BIOMARKERS OF HIV-1 INFECTION

To determine if IL-10 levels were correlated with biomarkers of HIV-1 infection, we determined the association between levels of IL-10 and viral load, $CD4^+$ T cell count, and the breadth and magnitude of immune responses. We used the Pearson's correlation to determine if there any association between IL-10 levels and biomarkers of HIV infection (see Figure 3.3.3). IL-10 plasma levels did not significantly correlate with viral load (Pearson's correlation= 0.08, p= 0.38; Figure 3.3.3A), $CD4^+$ T cell count (Pearson's correlation= -0.03, p= 0.77; Figure 3.3.3B), the breadth of immune responses (Pearson's correlation= -0.06, p= 0.53; Figure 3.3.3C) or the magnitude of immune responses (Pearson's correlation=0.06, p= 0.54; Figure 3.3.3D).

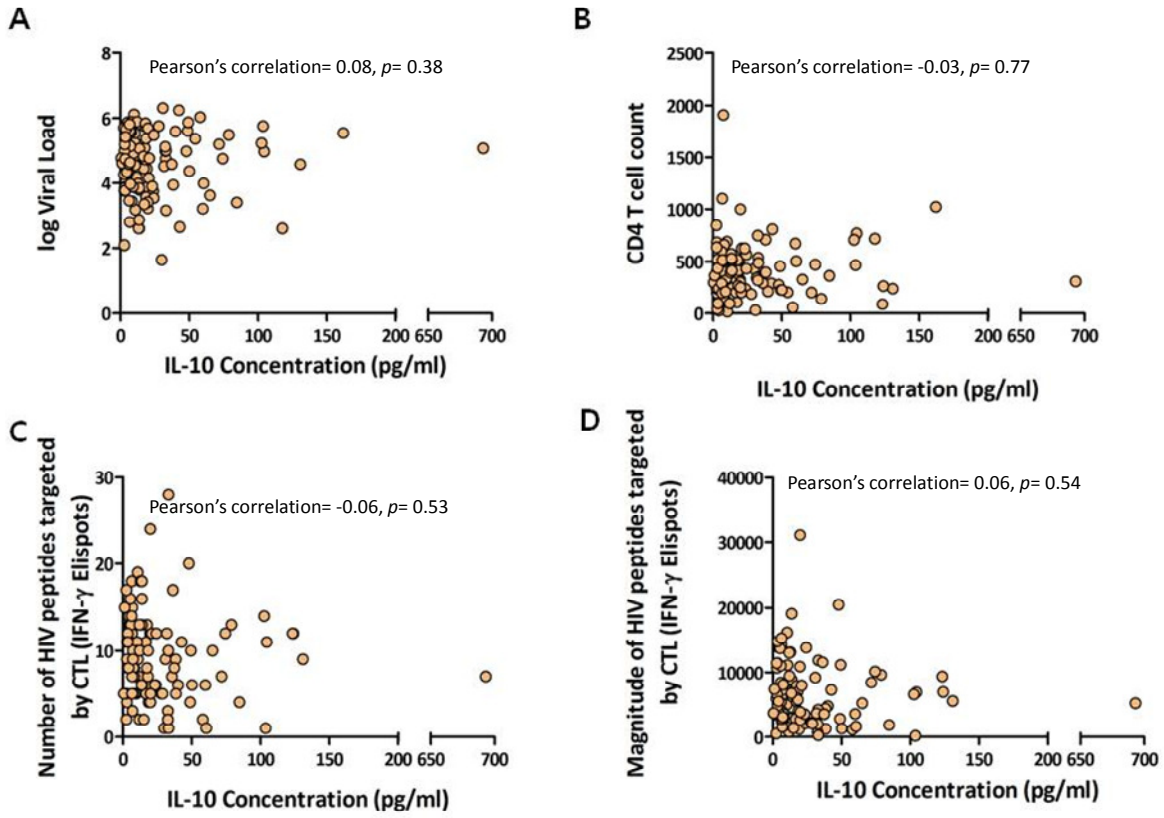

Figure 3.3.3 Correlation between IL-10 levels and biomarkers of HIV-1 infection. IL-10 did not correlate with any biomarkers of HIV-1 infection. (A) Correlation between IL-10 concentration and viral load (Pearson's correlation= 0.08, p= 0.38). (B) Correlation between IL-10 concentration and $CD4^+$ T cell count (Pearson's correlation= -0.03, p= 0.77). (C) Correlation between the breadth of immune responses and IL-10 concentration (Pearson's correlation= -0.06, p= 0.53). (D) Correlation between IL-10 concentration and the magnitude of immune responses (Pearson's correlation=0.06, p= 0.54).

3.3.3 ASSOCIATION AND CORRELATION BETWEEN IL-10 LEVELS AND OTHER CYTOKINES

As IL-10 is a major inhibitory immunoregulator, we wanted to determine the correlation between IL-10 levels and some pro-inflammatory cytokines, i.e. IFN-γ, IL-2, IL-6 and TNF-α (see Figure 3.3.4). There was significant positive correlation between the levels of each of the proinflammatory cytokines (IFN-γ, IL-2 and IL-6 and TNF-α) and IL-10 levels (p <0.0001, Spearman's r test).

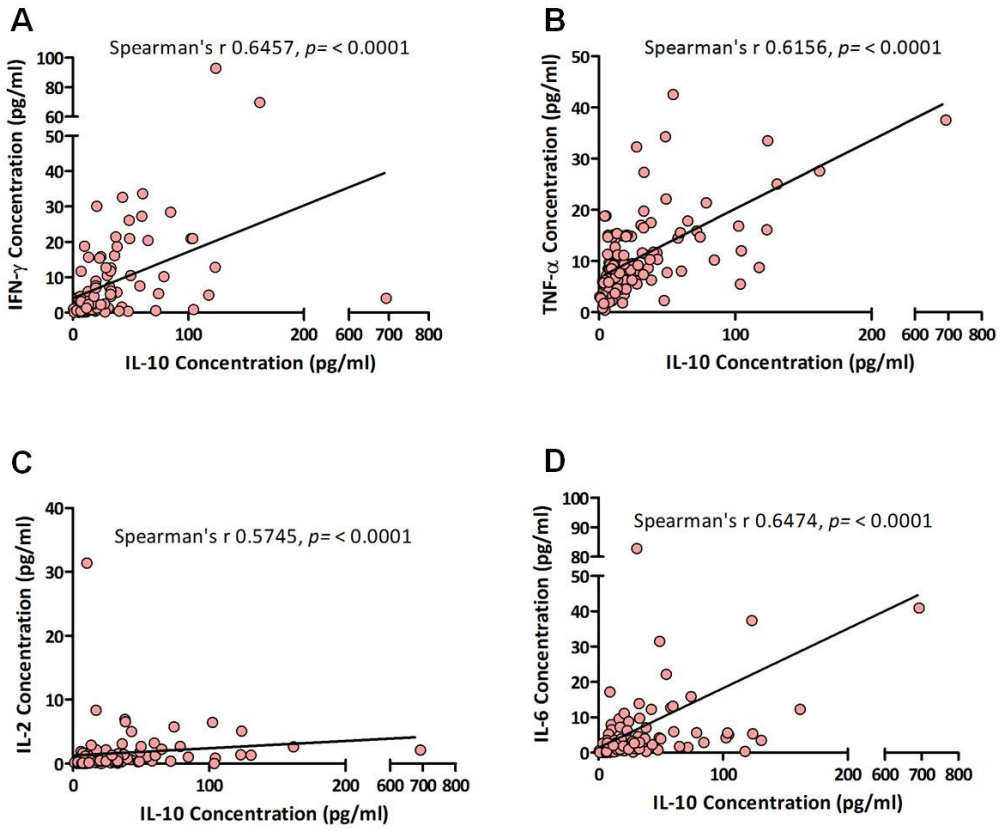

Figure 3.3.4 Correlation between IL-10 levels and pro-inflammatory cytokines. There was a significant correlation between IL-10 levels and levels of pro-inflammatory cytokines. (A) Correlation between IFN-γ and IL-10. (B) Correlation between TNF-α and IL-10. (C) Correlation between IL-2 and IL-10. (D) Correlation between IL-6 and IL-10.

We next investigated the proportions of measured cytokines based on *IL-10* genetic variant in the 112 individuals from the HPP Sinikithemba cohort. Figure 3.3.5A and B shows that overall, IL-10 dominated the measured plasma cytokine levels in this chronic HIV-1C setting irrespective of the *IL-10* genotype.

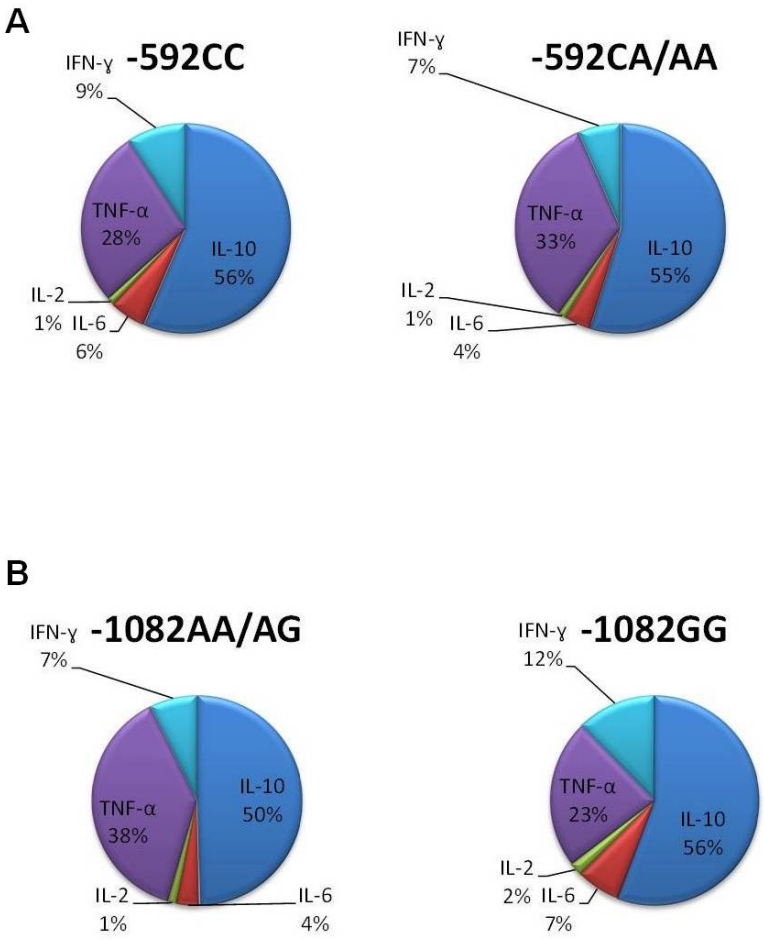

Figure 3.3.5 Cytokine expression during chronic HIV-1C infection. IL-10 dominated cytokine expression overall, regardless of *IL-10* genotype. (A) Proportion of cytokine expression, based on IL-10 -592 genotype. (B) Proportion of cytokine expression, based on *IL-10* -1082 genotype.

3.3.4 ASSOCIATION OF *IL-10* VARIANTS WITH BREADTH AND MAGNITUDE OF IMMUNE RESPONSES

In the LCMV mouse model of chronic viral infection, mice deficient in IL-10 showed an increased frequency of tetramer positive virus-specific $CD8^+$ T cells and IL-10 receptor blockade increased interferon-γ production by virus-specific $CD8^+$ T cells (Brooks et al., 2006, Ejrnaes et al., 2006). Therefore, we reasoned that in HIV-1 infection *IL-10* variants that influence IL-10 production and disease progression may also be linked with the magnitude (number of IFN-γ producing cells per million PBMCs) and the breadth (number of HIV peptides targeted by CTL) of HIV-1-specific immune response *in vivo,* as measured by IFN-γ ELISPOT.

We thus investigated the association between *IL-10* variants and the magnitude and breadth of $CD8^+$ T cell immune responses in 409 individuals from the HPP Sinikithemba Chronic Infection cohort. Figure 3.3.6A and 3.3.6B shows the association between the magnitude of immune responses based on the -592 and -1082 genotypes respectively. We found no significant association between the magnitude of HIV-1 specific immune responses and either the -1082 genotype (p= 0.44) or the -592 genotype (p= 0.17).

We then assessed the breadth of immune responses based on *IL-10* genotype. There was a significant association between the number of HIV peptides targeted and the -592 genotype (p= 0.007 see Figure 3.3.6C). We found no significant association between -1082 genotype and the number of HIV peptides targeted by CTL (p= 0.23; see Figure 3.3.6D). The low-IL-

10-producing -592AA group had a median of 12 HIV peptides versus 7 peptides targeted for the -592CC or -592CA genotypes (p= 0.002 and 0.004 respectively).

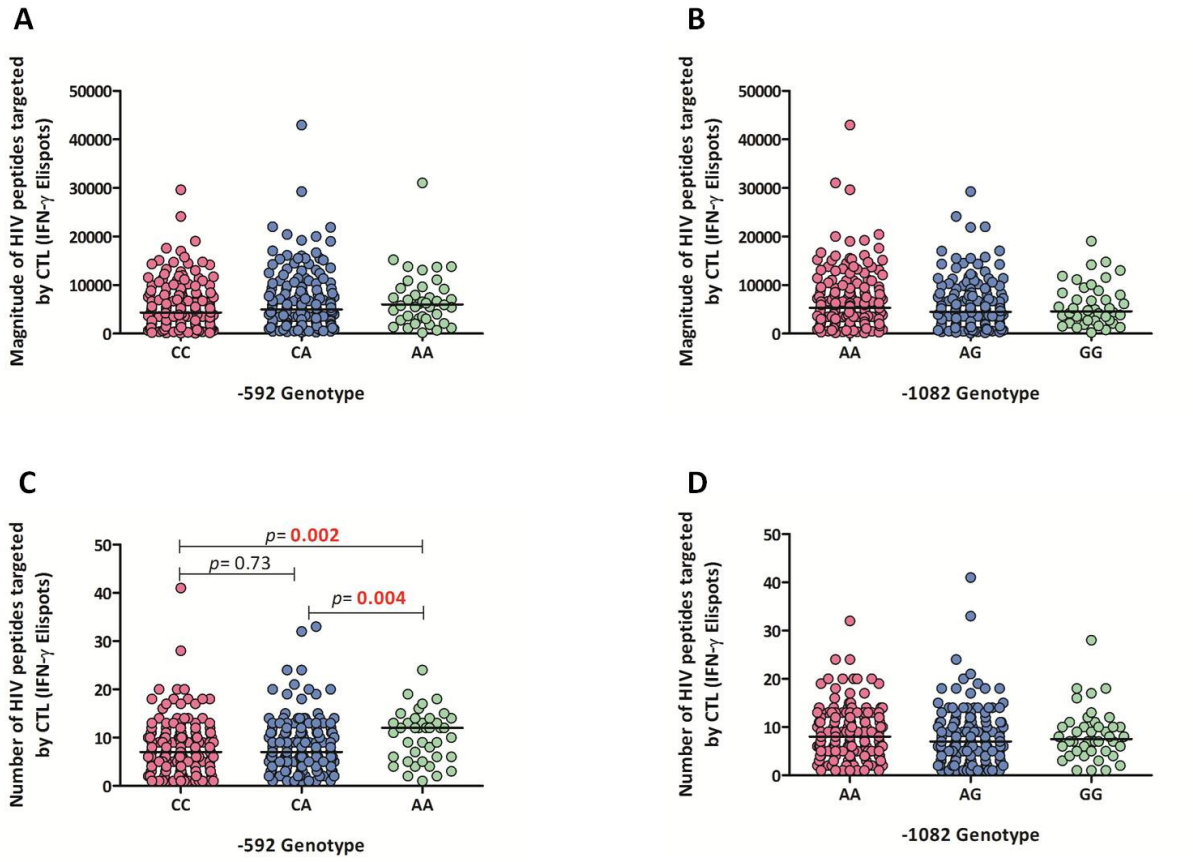

Figure 3.3.6 Magnitude and breadth of immune responses based on genotype. (A) The association between the magnitude of HIV peptides targeted by CTL (IFN-γ) and -592 genotype. There was no significant association noted (p= 0.17). (B) The association between magnitude of HIV peptides targeted by CTL (IFN-γ) and -1082 genotype. There was no significant association (p= 0.44). (C) The association between the number of HIV peptides targeted by CTL (IFN-γ) and -592 genotype. There was a significant association between the number of HIV peptides targeted by CTL and -592 genotype (p= 0.007). The -592AA group targeted a significantly larger number of HIV peptides as compared to the -592CC or 592CA groups (p= 0.002 and 0.004 respectively). (D) The association between the number of HIV peptides targeted by CTL (IFN-γ) and -1082 genotype. There was no significant association noted (p= 0.23).

We used the Wilcoxon rank sums test to determine if there was an association between any *IL-10* haplotype and either the breadth or magnitude of immune responses (see Table 3.3.6). We did not find any significant association between any *IL-10* haplotype and the magnitude of immune responses. However, we found that only the CGA haplotype had a significant association with the breadth of immune responses. Individuals with the CGA haplotype had a larger breadth of immune responses as compared to those individuals without the CGA haplotype (p= 0.02).

Table 3.3.6 Association between *IL-10* haplotype and the magnitude and breadth of immune responses in the HPP Acute Infection cohort

Haplotype		n	Median Magnitude (IQR)	*p*-value	n	Median Breadth (IQR)	*p*-value
AAT	No	9	0 (0 - 190)	0.81	9	3 (2 - 5)	0.71
	Yes	7	0 (0 - 200)		7	4 (2 - 4)	
CAA	No	16	0 (0 - 195)	-	16	4 (2 - 4)	-
	Yes	0	-		0	-	
CAT	No	8	0 (0 - 170)	1.00	8	4 (2 - 4)	0.91
	Yes	8	0 (0 - 195)		8	4 (1 - 5)	
CGA	No	7	0 (0 - 200)	0.54	7	2 (0 - 4)	**0.02**
	Yes	9	0 (0 - 140)		9	4 (3 - 5)	
CGT	No	12	0 (0 - 95)	-	12	4 (2 - 5)	-
	Yes	4	170 (70 - 750)		4	2 (2 - 3)	

We next investigated the relationship between *IL-10* haplotype and the breadth and magnitude of immune responses in the SK cohort of chronic infection (see Table 3.3.7). Here we found a significant association between the CAA haplotype and the magnitude of immune responses. We found that individuals without the CAA haplotype had a significantly larger magnitude of

CTL responses as compared to individuals with the CAA haplotype (p= 0.0005). We also found a significant association between the *IL-10* haplotype and the breadth of immune responses. Individuals without the CAA haplotype had a wider breadth of CTL responses as compared to individuals with the CAA haplotype (p= 0.005).

Table 3.3.7 Association between the *IL-10* haplotype and the breadth and magnitude of immune responses in the Sinikithemba Chronic Infection cohort

Haplotype		n	Median Magnitude (IQR)	*p*-value	n	Median Breadth (IQR)	*p*-value
AAT	No	189	966 (0 - 2105)	0.9752	167	8 (5 - 13)	0.1715
	Yes	259	980 (0 - 2428)		211	9 (6 - 14)	
CAA	No	436	1002 (0 - 2295)	**0.0005**	369	9 (6 - 14)	**0.0046**
	Yes	12	0 (0 - 0)		9	2 (1 - 6)	
CAT	No	204	938 (0 - 2215)	0.5910	169	9 (5 - 14)	0.8085
	Yes	244	1002 (0 - 2295)		209	9 (5 - 13)	
CGA	No	274	998 (0 - 2331)	0.6296	230	9 (6 - 14)	0.2748
	Yes	174	920 (0 - 2180)		148	8 (5 - 13)	
CGT	No	367	940 (0 - 2300)	0.8392	308	9 (6 - 14)	0.3512
	Yes	81	1000 (0 - 1945)		70	9 (5 - 11)	

3.4 DISCUSSION

In this part of the study, we wanted to investigate the mechanisms underlying the role of *IL-10* promoter polymorphisms on the pathogenesis of HIV-1 infection. For this part of the analysis we examined the association between *IL-10* genetic variants and IL-10 expression, biomarkers of disease progression, select pro-inflammatory cytokines and the breadth and magnitude of immune responses.

IL-10 promoter polymorphisms have been shown to affect IL-10 production (Edwards-Smith et al., 1999, Eskdale et al., 1998, Hutchinson et al., 1998, Turner et al., 1997). However, the effect of *IL-10* polymorphisms on IL-10 expression in the setting of chronic HIV-1 infection has not been previously investigated. Our analysis into the role of IL-10 in HIV-1C pathogenesis showed that these *IL-10* promoter polymorphisms that have been previously shown to be associated with differing levels of IL-10 expression, significantly associated with differential plasma IL-10 expression in an HIV setting. The *IL-10*-1082GG group, previously shown to be associated with increased IL-10 expression, showed a significantly higher median level of IL-10 expression as compared to the combined -1082AA/AG groups. Although we did not see an association with IL-10 expression and -592 group, the combined -592AA/CA groups, previously shown to be associated with decreased IL-10 expression, had a lower median level of IL-10 expression as compared to the -592CC group, however this was not significant. Also, the level of IL-0 expression may be influenced by HIV-infection itself or other co-infections (Crowley-Nowick et al., 2000). With regards to haplotype and IL-10 expression, we found that the CAT haplotype had a strong association. We found that

individuals without the CAT haplotype had a significant higher median IL-10 expression as compared to individuals with the CAT haplotype. The CAT haplotype is broken down as -592**C**/-1082**A**/-3575**T**. The observation is consistent with the observation that individuals carrying the -1082A allele had significantly lower levels of IL-10 as compared to individuals without the -1082A allele. We also found that overall, individuals without the CAT haplotype had significantly higher median levels of all cytokines measured, i.e. IL-2, IL-6, IFN- γ and TNF-α.

We investigated the role of IL-10 production with biomarkers of HIV-infection, such as viral load, $CD4^+$ T cell count, and the breadth and magnitude of immune responses. We did not find a correlation between plasma levels of IL-10 and any of these biomarkers of HIV-1 infection. As time of infection is not known in individuals in this cohort, we may be analysing biomarkers at different stages of infection. This may explain the lack of correlation between IL-10 expression and biomarkers of HIV-1 infection. Also, as there is an intricate network of cytokine expression, the expression of IL-10 may be affecting the expression of other pro- and anti-inflammatory cytokines, which may directly affect these biomarkers of HIV-1 infection. Also, the expression of cytokines in plasma may differ from the expression levels found within the mucosal or lymphoid tissue. We may have observed a different outcome, had we looked at the expression of cytokines in mucosal or lymphoid tissue, as it is in this tissue that HIV-1 predominantly replicates.

As the cytokine network is an intricate balance of signals and feedback, we wanted to determine the cytokine profile of select cytokines in an HIV setting of chronic infection. We sought to determine the association between IL-10 and the predominant Th1 cytokines IFN-γ, IL-2, IL-6 and TNF-α. We found that there was a significantly positive correlation between IL-10 and all of these cytokines measured. This suggests that during the chronic phase of HIV-1 infection, there is an upregulation of both pro- and anti-inflammatory cytokines. We investigated the proportions of cytokines, to determine the cytokine profile during chronic HIV-1 infection, and found that regardless of genotype, IL-10 expression seemed to dominate over the expression of the other cytokines. This suggests that as the production of pro-inflammatory cytokines increases, the production of IL-10 also increases, perhaps as a mechanism to reduce to inflammation and activation.

As IL-10 has been shown to play an important role in the immune response and immunoregulation, we then wanted to determine if *IL-10* variants had any association with the breadth (number of HIV peptides targeted by CTL) or magnitude (number of IFN-γ producing cells per million PBMCs) of HIV-1 specific immune responses *in vivo*, as measured by IFN-γ ELISPOT. *IL-10* genetic variants did not significantly associate with the magnitude of immune responses, however, the -592AA genotype did have a significant association with the number of HIV-specific peptides targeted by cytotoxic T lymphocytes (CTLs). This is consistent with mechanistic studies done on LCMV in the mouse model, where the removal or blockade of IL-10 enhanced T-cell immune responses (Brooks et al., 2006, Ejrnaes et al., 2006). The -592AA genotype has been previously shown to be associated with low IL-10 production, and in our study we found that individuals with this genotype tended towards a

lower median level of IL-10 expression, however this was not significant. The resulting lower IL-10 levels may allow for increased expression of HLA Class I and II molecules on the surface of cells. This therefore increases pathogen-derived peptide presentation on the cell surface of infected cells, which can be recognised by $CD8^+$ T lymphocytes. However, as IL-10 levels did not correlate with biomarkers of HIV-1 infection, *IL-10* genetic variants may contribute to the quality of immune responses via a complex pathway that has yet to be elucidated. The CGA and CAA haplotypes had a significant association with breadth and magnitude of immune responses. We found that individuals with the CGA haplotype in the HPP Acute Infection cohort had a significantly larger breadth of immune responses as compared to individuals without the CGA haplotype. In the Sinikithemba Chronic Infection cohort, individuals without the CAA haplotype had a significantly greater magnitude of immune responses, as well as a significantly larger breadth of immune responses, as compared to individuals with the CAA haplotype. Individuals with the CAA haplotype had a lower median IL-10 expression, however this was not significant. This suggests that lower levels of IL-10 may favour a greater magnitude of immune responses, and a larger breadth of peptides targeted by CTL.

Overall, our data suggest an association between *IL-10* promoter genotypes with plasma IL-10 levels, a predominance of the anti-inflammatory IL-10 over proinflammatory cytokines in the plasma of HIV-1 infected individuals and an effect of *IL-10* polymorphisms on the breadth but not the magnitude of $CD8^+$ T-cell immune response. Additional mechanistic studies will also be required in order to fully understand how best to target the IL-10 pathway for effective immunotherapy or a vaccine.

Chapter 4

The role of *IL-10* variants and IL-10 blockade on $CD4^+$, $CD8^+$ T-cell and B cell activation and $CD4^+$ T-cell proliferation

Chapter 4: The role of *IL-10* variants and IL-10 blockade on $CD4^+$, $CD8^+$ T-cell and B-cell activation and $CD4^+$ T-cell proliferation

4.1 INTRODUCTION

The underlying mechanisms on the role of IL-10 and its genetic variants in HIV pathogenesis have not been well studied despite the evidence of their importance from the genetic association studies. Previous studies have shown that *IL-10* promoter polymorphisms play a role in HIV-1 susceptibility and pathogenesis (Erikstrup et al., 2007, Naicker et al., 2009, Shin et al., 2000). However, the underlying mechanisms that control the effect of these polymorphisms are not well studied, particularly in the African setting of high incidence and prevalence and where viral genetic subtypes differ from the Western countries.

HIV-1 infection severely compromises the immune system, resulting in dramatic $CD4^+$ T cell loss. Previous studies have shown that individuals that are able to control HIV replication, i.e. controllers, maintain high levels of IL-2-secreting $CD4^+$ T cells, which proliferate when exposed to HIV peptides (Emu et al., 2005, Rosenberg et al., 1997). Generalised T cell activation has been implicated in contributing to immunodeficiency in HIV-1 infection. Many studies have demonstrated that elevated T cell activation levels are associated with a rapid HIV disease progression and CD4 decline in antiretroviral naïve individuals (Deeks et al., 2004, Giorgi et al., 1999, Giorgi et al., 2002, Liu et al., 1998, Liu et al., 1997, Sousa et al., 2002).

Generalised T cell activation due to chronic HIV stimulation may drive excessive T cell proliferation, expansion and death, eventually leading to immunologic exhaustion of the regenerative capacity of the immune system (Hazenberg et al., 2000, McCune, 2001, Pantaleo et al., 1997).

IL-10 has been implicated in modulating T cell activation and has been shown to suppress T cell proliferation, as well as blocking pro-inflammatory cytokine production. IL-10 acts on T helper cells to regulate Th1 cell proliferation and differentiation (Filippi and von Herrath, 2008). In a study by Torheim *et al* (Torheim et al., 2009), it was demonstrated that a small subset of HIV-specific T cells that secrete IL-10 had the ability to inhibit proliferation of other T cells. Previous studies have also demonstrated that individuals with severely compromised T helper cell function have higher levels of IL-10 (Clerici et al., 1994, Ostrowski et al., 2001). Studying the underlying mechanisms of *IL-10* promoter polymorphisms can assist in better understanding the role of IL-10 in T helper cell function.

Blockade of IL-10 and the IL-10 receptor has been shown to result in resolution of a chronic viral infection (Brockman et al., 2009). Brockman *et al* (2009) studied the effect of IL-10 blockade on HIV- specific T cell function. They demonstrated that the blockade of IL-10 restored HIV-specific $CD4^+$ T cell proliferation, as well as antigen-specific $CD8^+$ T cell proliferation. Mechanistic studies of lymphocytic choriomeningitis virus (LCMV) in the mouse model showed that IL-10 blockade or gene knock-out resulted in enhanced T cell immune responses (Brooks et al., 2006, Ejrnaes et al., 2006). As a result this led to rapid elimination of LCMV and the development of antiviral memory T cell responses. IL-10 has also been shown to enhance detrimental deletion of dendritic cells by natural killer cells (Alter et al., 2010), adding to severe immune dysfunction in chronic HIV-1 infection.

Our data suggests that IL-10 and its genetic variants may play a role in CD4 decline; IL-10 may also dominate the cytokine profile during chronic infection favouring an anti-inflammatory profile; as well as play a role in the breadth of immune responses. In this study,

we sought to determine whether the previously observed associations of *IL-10* genetic variants and IL-10 on chronic HIV-1 pathogenesis are mediated via impact on $CD4^+$ T cell, $CD8^+$ T cell and B cell activation and proliferation. Specifically, the following markers were measured in $CD4^+$ and $CD8^+$ T cells, to determine if there is an association between expression of these markers and *IL-10* genetic variants: CD38, CD95, Ki67, HLA-DR and PD-1. The following markers were measured on B cells: CD38, CD95, IgG, Ki67 and PD-1. The CD38 molecule acts as a receptor that controls adhesion and signaling in leukocytes (Deaglio et al., 2001). The CD95 receptor has a significant role in the immune system, as it is responsible for cell death signaling by apoptosis, which plays a significant role in the life and function of immune system cells (Krammer, 2000). The expression of the Ki67 protein is a marker for proliferating cells, and is used to determine the growth-fraction of a given cell population (Scholzen and Gerdes, 2000). HLA-DR molecules are responsible for presenting antigen, on the surface of cells, to T helper cells. This presentation of antigen then results in the initiation of immune responses (Bottazzo et al., 1983). HLA-DR is used as a marker for immune activation during HIV-1 infection (Giorgi and Detels, 1989, Kestens et al., 1992, Liu et al., 1997, Prince and Jensen, 1991, Salazar-Gonzalez et al., 1985). The PD-1 molecule is a marker of T cell exhaustion in the face of chronic antigenic stimulation, a cell-death inducer, and is involved in programmed cell death (Day et al., 2006, Ishida et al., 1992). Immunoglobulin G (IgG) molecules are antibody molecules indicate the induction of the secondary immune response on B cells, they have the ability to inactivate viruses, and activate the classical complement pathway on various cell types (Meulenbroek, 1996, Pier, 2004).

4.2 MATERIALS AND METHODS

4.2.1 STUDY POPULATION

For the activation and proliferation assays we focused on the HPP Sinikithemba Chronic Infection cohort. We analysed the activation of $CD4^+$ T cells, $CD8^+$ T cells and B cells in a subset of 63 individuals. These individuals were chosen based on extreme genotypes (based on the dominant/recessive pattern of the SNP variants) to increase our chances of detecting a difference between groups, as our sample size was restricted due to sample availability and viability. Specimen collection and preparation was performed on fresh blood samples taken at scheduled visits. Blood samples were collected in ACD tubes and PBMCs were isolated by density gradient centrifugation no longer than 4 hours after phlebotomy was performed. The PBMCs were then directly processed for flow cytometry.

The CFSE proliferation assays were performed on a subset of individuals from the HPP Sinikithemba cohort. Blood samples were taken at scheduled visit, and PBMCs were isolated. PBMCs were frozen down and stored in liquid nitrogen until assays were performed. As the assays were performed on samples that had been thawed, the viability of the samples varied. We were able to assess IL-10 blockade in 31 out of the intended subset of 40 individuals from the SK cohort. We simultaneously measured cytokine expression after IL-10 blockade in 40 individuals.

As baseline samples were not available for all individuals included in the blockade assays, we included timepoints where samples were available. We also measured plasma cytokine levels in the 40 individuals included in the blockade analysis.

4.2.2 FLOW CYTOMETRIC DETECTION OF ACTIVATION MARKERS

Levels of $CD8^+$ T cell, $CD4^+$ T cell and B cell activation were assessed by flow cytometry. The following monoclonal antibody combinations were used: anti-CD3 Pac-blue, anti-CD38 PE-Cy7, anti-HLA-DR ACP-Cy7, anti-CD95 PE, anti-CD19 Alexa-700, anti-IgG PE-Cy5, anti-PD-1 APC, anti-Ki67 FITC (Becton Dickinson, San Jose, CA, USA) and anti-CD4 Qdot605 and anti-CD8 Qdot655 (Invitrogen, Life Technologies, Grand Island, NY, USA). All markers were assessed by surface staining, except Ki67, which was stained after permeabilizing cells with PERM B (Invitrogen, Life Technologies, Grand Island, NY, USA). Samples were analysed on a LSRII flow cytometer (Becton Dickinson, San Jose, CA, USA). For all activation markers percentages of positive cells were analyzed within T cell and B cell subsets. Activation marker co-expression profiles were determined using the Flowjo software (Tristar, Ashland, OR, USA). The gating strategy is shown in Figure 4.2.1.

PBMCs were used for the detection of activation markers, 1 million cells/tube. 2 ml of PBS was added to the samples which were then centrifuged at 1,700 rpm for 7 minutes. After centrifugation, the supernatant was discarded, and residual liquid was blotted on gauze without turning over the tubes. The FMO stain was added to all tubes as follows: 1.5 µl of CD3 PacBlue, 0.4 µl of CD4 Qdot605, 0.4 µl of CD8 Qdot655, and 4 µl of CD19. The surface stain was added to the tubes as follows: 6 µl of HLA-DR APC-Cy7, 6 µl of CD38 PE-Cy7, 10 µl of

CD95 PE, 12 µl of PD-1 APC and 12 µl of IgG Pe-Cy-5 (or Ki67 FITC in B cells). These samples were then incubated at 4^{o}C in the dark, for 15 minutes. Samples were then washed by adding 2 ml PBS and then centrifuging at 1,700 rpm for 7 minutes. The supernatant was decanted as before. Finally, 200 µl of PBS was added to resuspend the beads and this was stored at 4^{o}C in the dark until acquired on the LSRII Flow Cytometer (Becton Dickinson, San Jose, CA, USA).

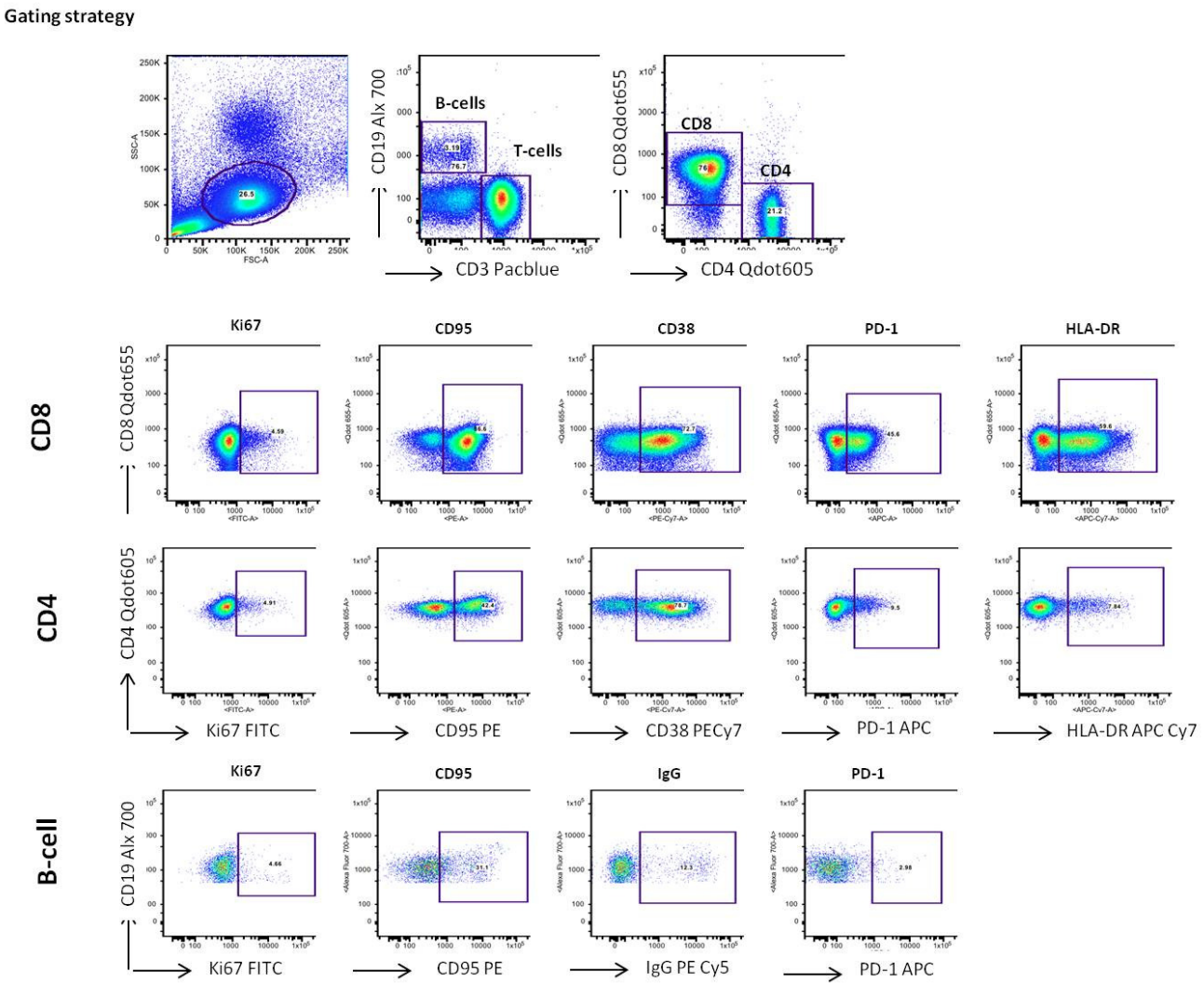

Figure 4.2.1 Gating strategy used for detection of activation markers by flow cytometry

4.2.3 IL-10 BLOCKADE ON CD4$^+$ T CELLS: CFSE ASSAYS AND SUPERNATANTS FOR CYTOKINE PROFILES

To determine whether IL-10 or its genetic variants played a role in CD4$^+$ T cell proliferation, IL-10 blockade was performed and CFSE assays, and supernatants were collected and measured. IL-10 blockade assays were performed on frozen PBMCs from 40 individuals from the Sinikithemba Chronic Infection cohort, of which 31 had positive results for proliferation. Cytokines were measured in all 40 individuals. The percent of proliferating CD4$^+$ T cells were determined using the Flowjo software (Tristar, Ashland, OR, USA). The gating strategy is shown in Figure 4.2.2.

PBMC samples were prepared prior to proliferation and stimulation assays. PBMC samples were thawed by following a standard operating procedure (SOP) as follows: samples were thawed (incompletely) in a water bath set to 37^{o}C for about 1 minute. Samples were added to a 15 ml tube containing 8 ml R+ (a solution composed of 500 ml RPMI media, 10 ml HEPES buffer solution, 5.5 ml of Penicillin Streptomycin and 5.5 ml of l-glutamine). 1 ml of FCS (fetal calf serum) and 30 µl of DNAse was added to the vials. Samples were mixed by gently inverting the tubes. The tubes were then centrifuged at 1,500 rpm for 7 minutes at 4^{o}C. Supernatant was discarded after centrifugation and the pellet was resuspended in a total of 3 ml R+ and centrifuged again at 1,500 rpm for 7 minutes at 4^{o}C. Cell count was performed manually using a microscope.

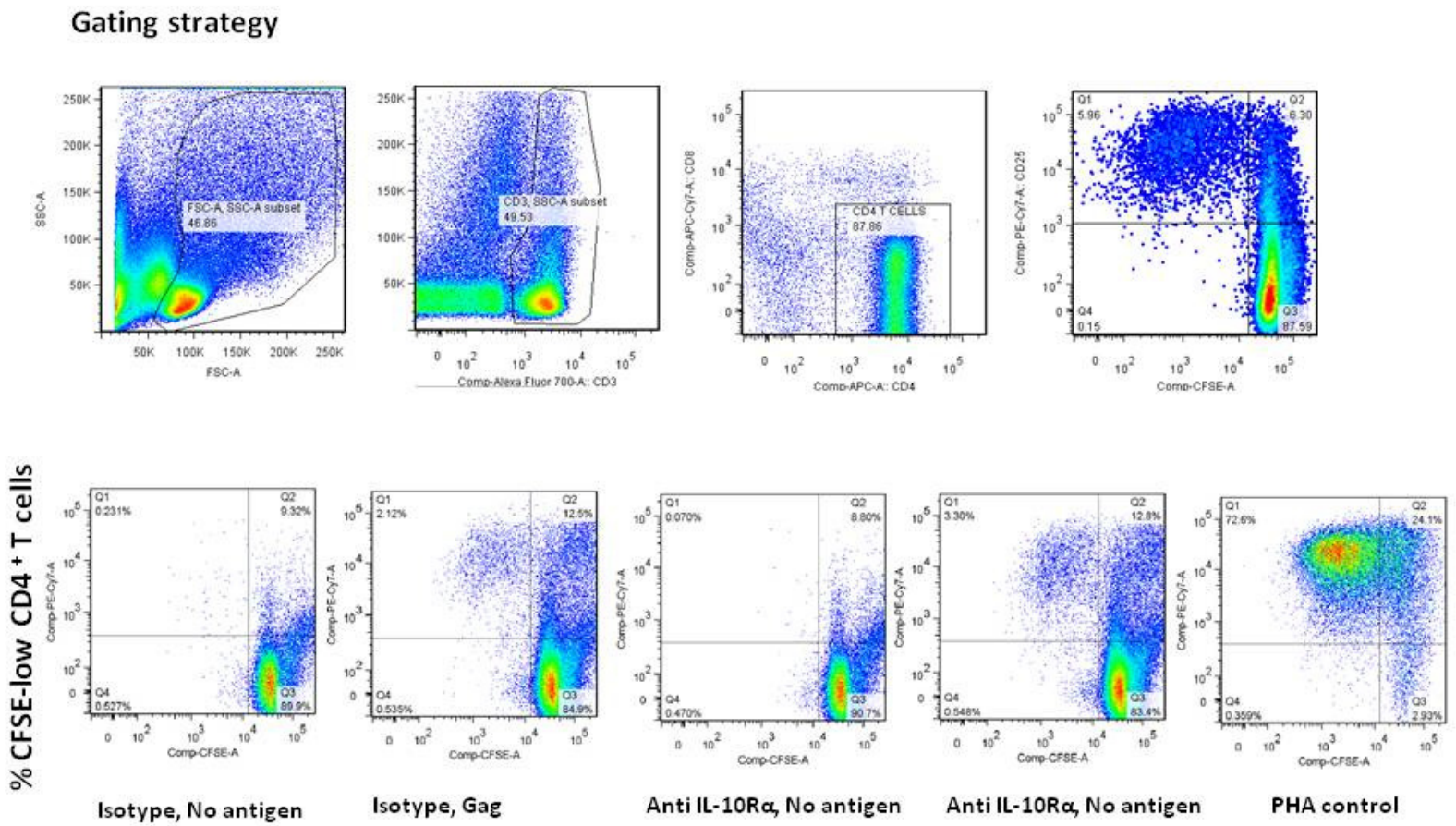

Figure 4.2.2 Gating strategy used to measure CD4$^+$ T cell proliferation after IL-10 blockade

The PBMCs were then $CD8^+$ T cell depleted, so that only $CD4^+$ T cells were used for the assays. CD8+ T cell depletion was done using the CD8 Dyna Beads (Invitrogen Life Technologies, Grand Island, NY, USA). The manufacturer's protocol was followed. Briefly, 25 µl of the beads were added for every 10 million cells used. Beads were washed by adding 1 ml PBS with 1% FCS prior to samples addition then placing in the magnetic tube rack for about 1 minute. The beads attracted to the magnet and the supernatant was removed leaving behind the beads on the side of the tube. The tubes were removed and the beads resuspended in PBS with 1% FCS proportional to the amount used. This was then added to the samples and placed back in the magnetic rack for 20 minutes at 4^oC. After 20 minutes the supernatant (containing the $CD4^+$ T cells) was placed into a clean tube, leaving behind the $CD8^+$ T cells captured on the Dyna beads. Cells were then manually counted and washed again, then resuspended. At this point the samples that were prepared for Luminex were resuspended in 2 ml R10 HAB (a mix of R+ and Human antibody serum, where 10% of the solution is HAB) for 3 hours at 37^o C, until blockade and stimulation.

Samples for the CFSE assay were resuspended in 1 ml PBS/CFSE mix (0.25 µl of CFSE was used per millilitre of PBS). After adding the PBS/CFSE mix, samples were immediately mixed and placed in an incubator for exactly 7 minutes at 37^oC. After 7 minutes 1 ml FCS and 8 ml R+ were added, to bring the volume up to 10 ml. This was centrifuged at 1,500 rpm for 7 minutes at 4^oC, and the supernatant was then discarded. The PBMC pellet was then washed again by adding 10 ml R+ and centrifuging for at 1,500 rpm for 7 minutes at 4^oC, and the supernatant was then discarded. The remaining pellet was then resuspended in 2 ml R10 HAB.

The cells were then rested for 3 hours in an incubator at 37^{o}C. This final volume would allow us to measure four different conditions with 1 tube of 500 µl each.

The four different conditions of IL-10 blockade and stimulation were done using the following: IL-10 receptor blockade with the human IL-10Rα antibody, the isotype control was IGg1 and the Gag antigen for stimulation. The four conditions were prepared as follows:

1) 5 µl IGg1 with no antigen stimulation;
2) 5 µl IGg1 with 5 µl Gag for stimulation;
3) 5 µl IL-10Rα with no antigen stimulation; and
4) 5 µl IL-10Rα with 5 µl Gag for stimulation.

Cells for both the Luminex and CFSE assays were stimulated using the same conditions as described above. The 2 ml of sample for the Luminex and CFSE assays was split into 4 tubes containing 500 µl of sample each, and the respective antibody, isotype or stimulation was added to the respective tube. After stimulation using the above conditions, the samples were treated differently for either the Luminex assays or the CFSE assays, as described below.

For the Luminex assays the stimulated cells were then vortexed gently and incubated for 48 hours at 37^{o}C. After 48 hours cells were transferred to microcentrifuge tubes and centrifuged at 5,000 rpm for 5 minutes at room temperature to pellet the cells. After centrifugation the supernatant was collected and split into two tubes for further use, and the cells were resuspended in 300 µl of RLT Buffer/Betamercaptoethanol (10µl of betamercaptoethanol was added for every 1ml of RLT Buffer used). The cells were then stored at -80^{o}C for future use.

Cytokines were measured in the supernatant using Luminex methodology, as previously described in Chapter 3.2.2. We measured 6 cytokines, i.e. IFN-γ, IL-2, IL-6, IL-10, IL-13, and TNF-α.

For the CFSE assays, the samples were incubated at 37^{o}C for 7 days after stimulation. After 7 days, 100 µl was removed from the isotype-no-antigen tube and put in a clean tube to use for the compensation setup for flow cytometry. 3 ml of PBS/1%FCS was added to all tubes and then centrifuged at 1,500 rpm for 7 minutes at 4^{o}C. Supernatant was discarded and the cells were then stained before analysing on the flow cytometer. Staining antibodies were used as follows: 5 µl of CD4 APC; 5 µl of CD25 Pe-Cy7; 1.5 µl of CD8 APC-Cy7; and 1.5 µl of CD3 Alexa 700. After the staining antibodies were added, the cells were incubated at 4^{o}C for 20 minutes in the dark. After 20 minutes of incubation, samples were washed by adding 3 ml PBS/1%FCS and centrifuged at 1,500 rpm for 7 minutes at 4^{o}C. The supernatant was then discarded and 200 µl of 4% PFA (Paraformaldehyde) was added to fix the cells so that they could be analysed on the flow cytometer. This was then left in the dark at room temperature for 20 minutes. After 20 minutes incubation, the samples were washed again by adding 3 ml PBS/1%FCS and centrifuged at 1,500 rpm for 7 minutes at 4^{o}C. The supernatant was discarded and the pellet was then resuspended in 200 µl PBS/1%FCS, ready for analysis. The samples were kept at 4^{o}C in the dark until they were acquired on the LSRII Flow Cytometer (Becton Dickinson, San Jose, CA, USA).

4.2.4 PLASMA CYTOKINE PROFILING BY LUMINEX METHODOLOGY

Cytokines were measured in the plasma of samples with matched timepoints as the PBMCs used in the IL-10 blockade assays. These plasma samples were prepared routinely in the lab at scheduled visits. The plasma was removed from whole blood by centrifugation and stored in liquid nitrogen until used. We measured six cytokines using Luminex methodology. We measured the plasma concentration of IFN-γ, IL-2, IL-6, IL-10, IL-13, and TNF-α, using methods described in Chapter 3.2.2.

4.2.5 STATISTICAL ANALYSIS

Both univariate and multivariate analyses were performed in assessing the association between activation markers and *IL-10* genetic variants. The Kruskal-Wallis test was used to compare activation markers between genotypes and haplotypes. Subsequent analysis of difference between any two genotypes/haplotypes was performed using Wilcoxon rank sum test. Rank analysis of covariance was used to adjust for the effect of HIV viral RNA while comparing the activation markers between the genotypes and haplotypes. A 5% level of significance in the univariate analyses was used to consider variables for the multivariate analyses. For the IL-10 blockade assays, in determining whether there is any difference in cell proliferation after blockade, a paired non-parametric test, namely the Wilcoxon signed rank test was performed on the difference between isotype and IL-10Rα. With cytokine profiling after IL-10 blockade a similar analysis was undertaken by performing a paired non-parametric test, i.e. the Wilcoxon signed rank test, on the difference between the isotype and IL-10Ra within each cytokine. For the matched timepoint plasma cytokine profiles, the Wilcoxon rank sums test was used to compare the *IL-10* genotypes -592 and -1082, within each of the cytokines. The

Wilcoxon ranks sums test was used to determine whether the presence of a haplotype was associated with the following: cell proliferation after IL-10 blockade, cell proliferation and viral load, cytokine production after IL-10 blockade and plasma cytokine expression.

4.3 RESULTS

4.3.1 ASSOCIATION BETWEEN BIOMARKERS OF HIV INFECTION AND MARKERS OF ACTIVATION ON $CD4^+$ T CELLS, $CD8^+$ T CELLS AND B CELLS

We wanted to determine if there was an association between biomarkers of HIV infection and any activation marker on $CD4^+$ T cells, $CD8^+$ T cells and B cells. To do this we fitted Linear regression models to $CD4^+$ T cell count, viral load, and the number and magnitude of HIV peptides targeted by CTL. For each cell subset unadjusted analyses were performed, and then, adjusted models were created by including factors which were significant at a 20% level of significance. Figure 4.3.1 shows the relationship between the expression of activation markers on $CD8^+$ T cells and $CD4^+$ T cell count. In the adjusted analyses CD38 and CD95 were significantly negatively associated with $CD4^+$ T cell count. For every one percent increase in the expression of CD38, $CD4^+$ T cell count decreased by approximately 3 cells/µl (p= 0.04) and decreased by almost 5 cells/µl for every one percent increase in CD95 expression (p= 0.04).

We next investigated the relationship between the expression of activation markers on $CD4^+$ T cells and $CD4^+$ T cell count (see Figure 4.3.2). Again CD38 and CD95 expression had a significant negative association with $CD4^+$ T cell count. With every one percent increase in expression of CD38, there was a decrease in almost 5 cells/µl (p= 0.02), and a decrease of almost 4 cells/µl for CD95 expression (p= 0.05).

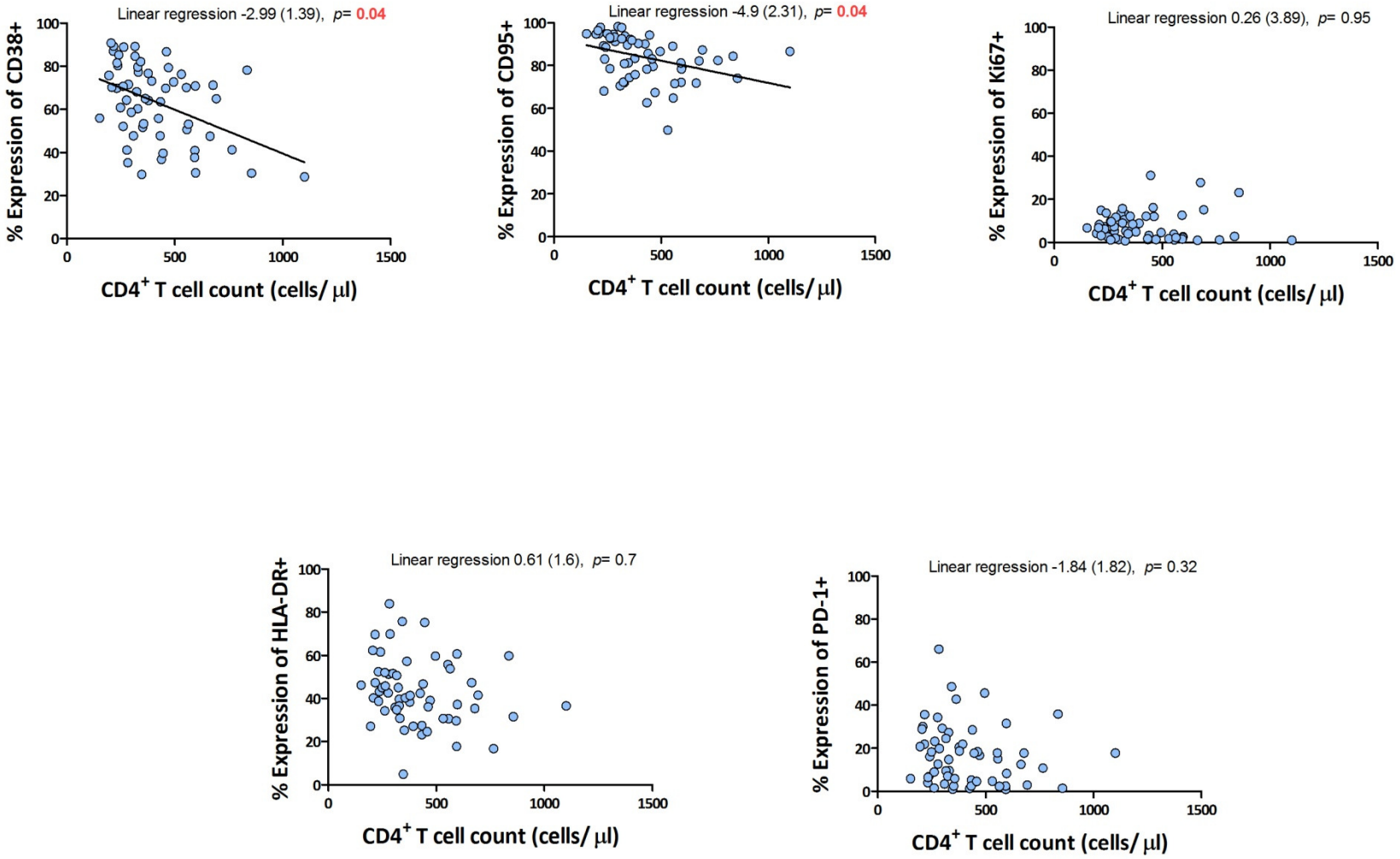

Figure 4.3.1 The relationship between activation markers on CD8$^+$ T cells and CD4$^+$ T cell count. CD38 and CD95 expression had a significant negative association with CD4 T cell count. The Standard Error is shown in parenthesis.

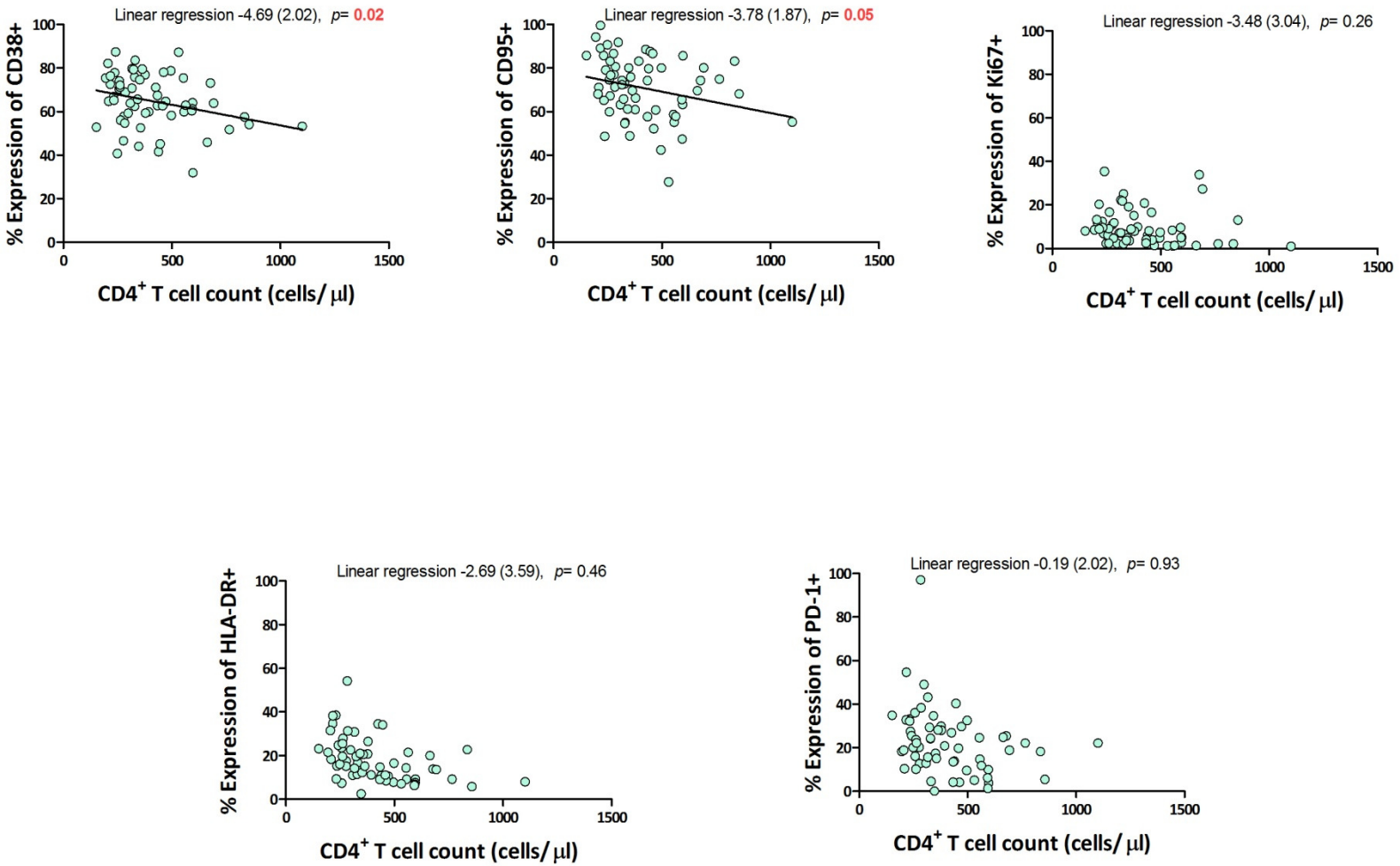

Figure 4.3.2 The relationship between activation markers on CD4$^+$ T cells and CD4$^+$ T cell count. CD38 and CD95 expression had a significant negative association with CD4 T cell count. The Standard Error is shown in parenthesis.

The relationship between the expression of activation markers on B cells and $CD4^+$ T cell count is shown in Figure 4.3.3. CD95 was the only marker that showed a significant negative association with $CD4^+$ T cell count. For every one percent increase in the expression of CD95, there was a decrease of 3 cells/µl (p= 0.03).

We next investigated the relationship between the expression of activation markers on $CD4^+$ T cells, $CD8^+$ T cells, and B cell and viral load. Figure 4.3.4 shows the relationship between markers of activation on $CD8^+$ T cells and viral load. CD38 was the only marker of $CD8^+$ T cell activation that showed a significant positive association with viral load. For every one percent increase in expression of CD38, there was a 0.02 log increase in viral load (p= 0.02).

Figure 4.3.5 shows the relationship between the expression of markers of activation on $CD4^+$ T cells and log viral load. We found no significant association between any activation markers on $CD4^+$ T cells and log viral load.

Figure 4.3.6 shows the association between the expression of activation markers on B cells and log viral load. CD95 was the only activation marker on B cells that showed a significant positive assocation with log viral load. For every one percent increase in the expression of CD95, there was a 0.03 log increase in viral load (p= 0.004).

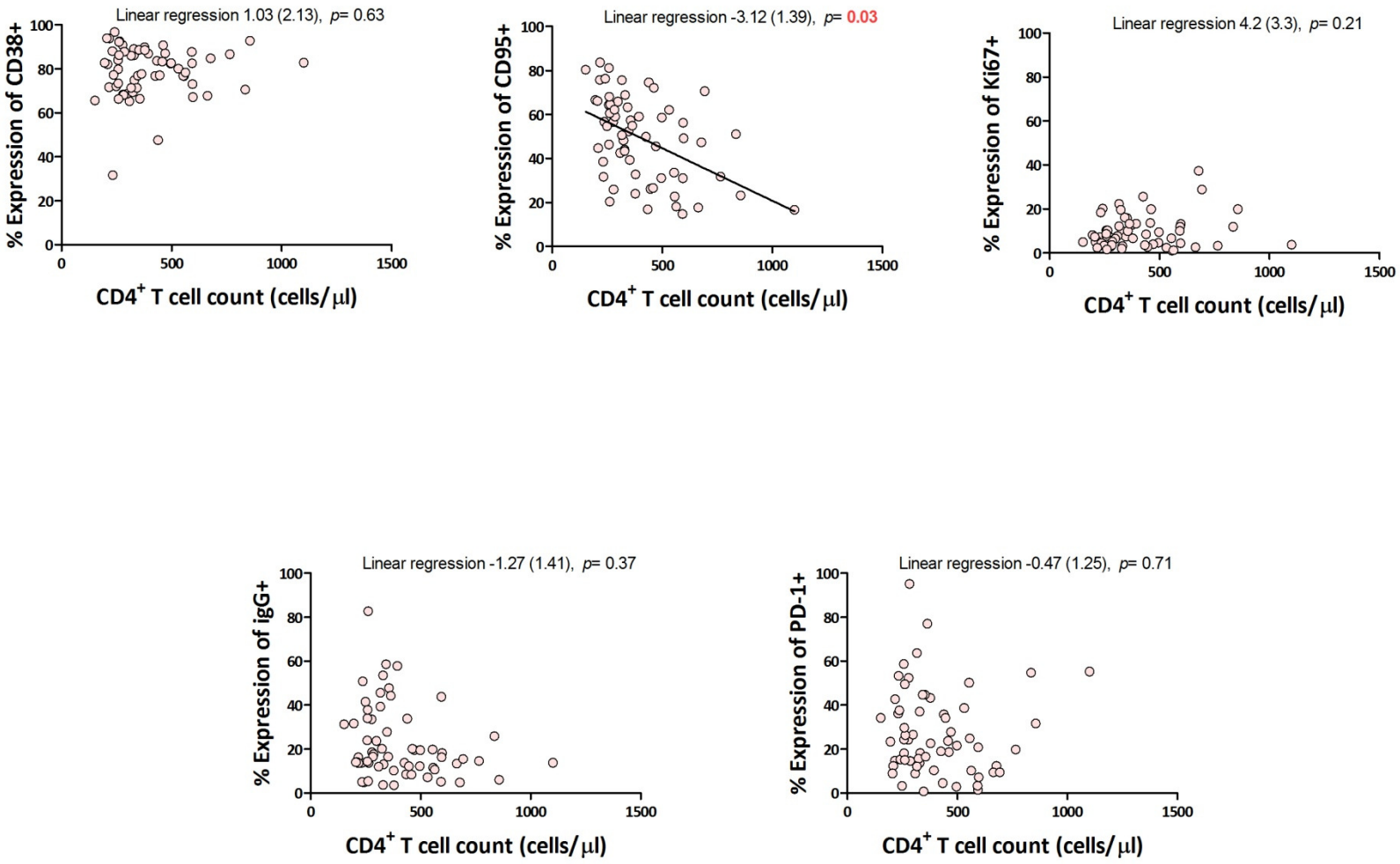

Figure 4.3.3 The relationship between markers of activation on B cells and CD4$^+$ T cell count. CD95 had a significant negative association with CD4$^+$ T cell count. The Standard Error is shown in parenthesis.

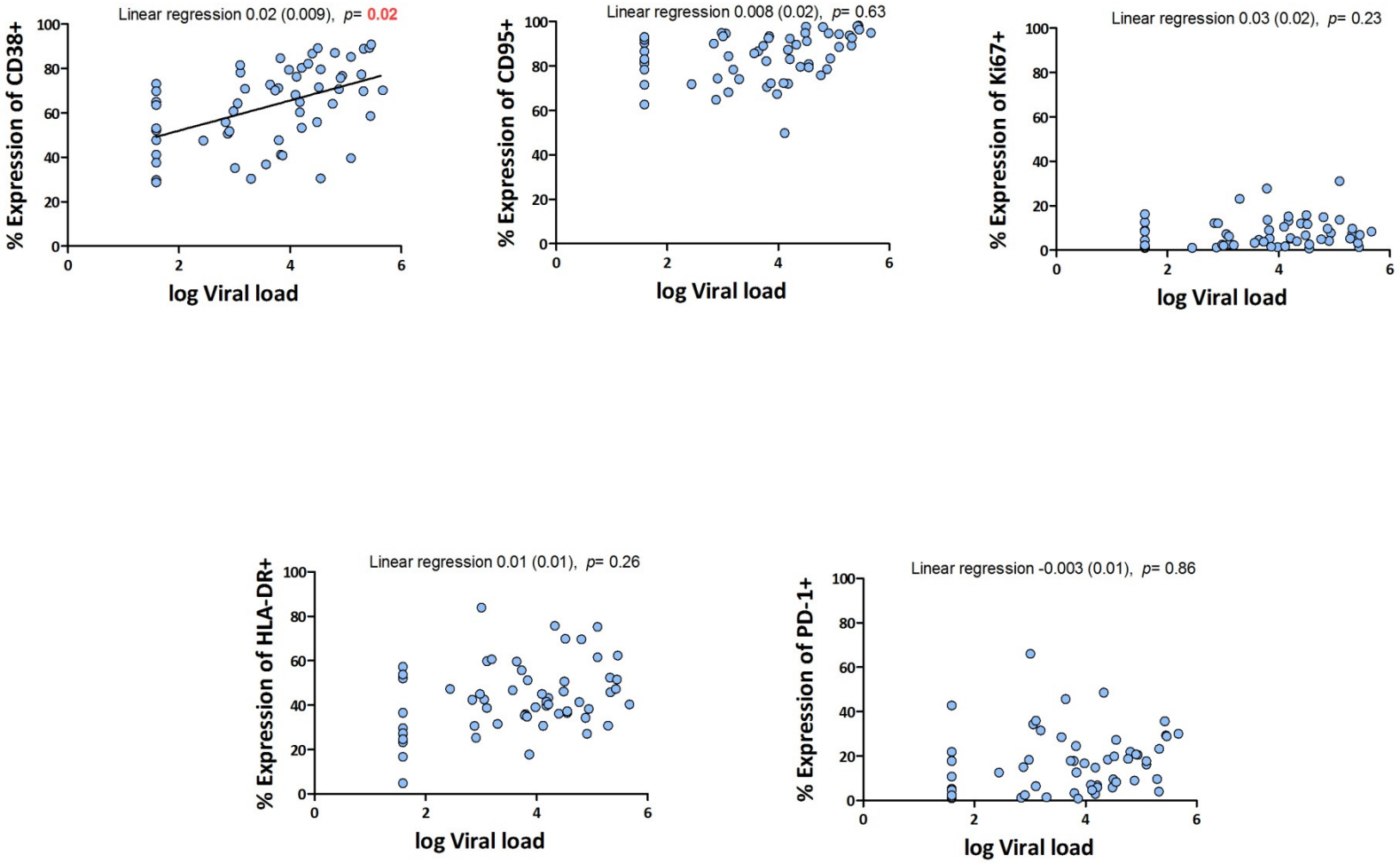

Figure 4.3.4 The relationship between markers of activation on CD8$^+$ T cells and viral load. CD38 had a significant positive association with log viral load. The Standard Error is shown in parenthesis.

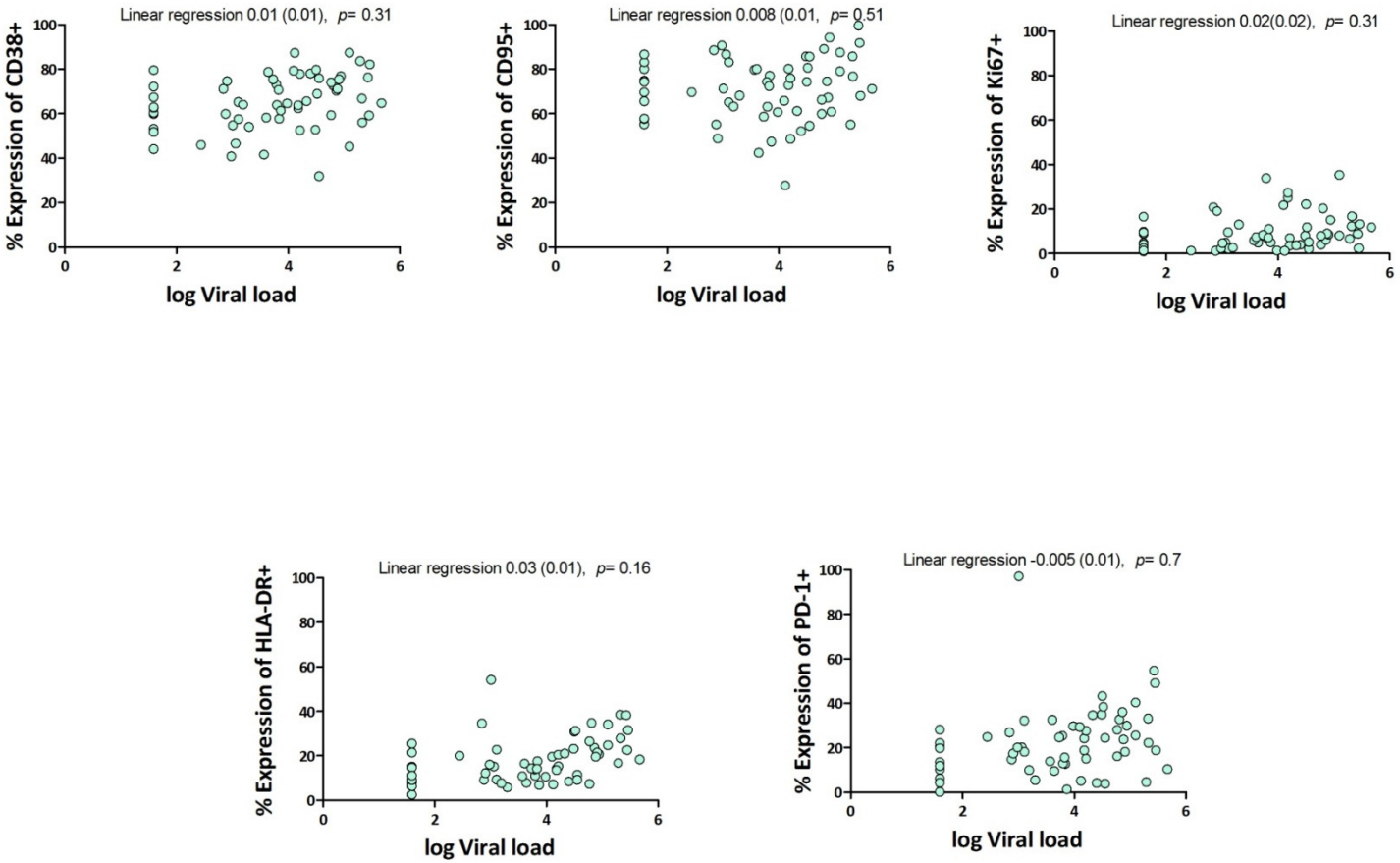

Figure 4.3.5 The relationship between markers of activation on CD4$^+$ T cells and log viral load. There was no significant association between the expression of any markers of CD4$^+$ T cell activation and log viral load. The Standard Error is shown in parenthesis.

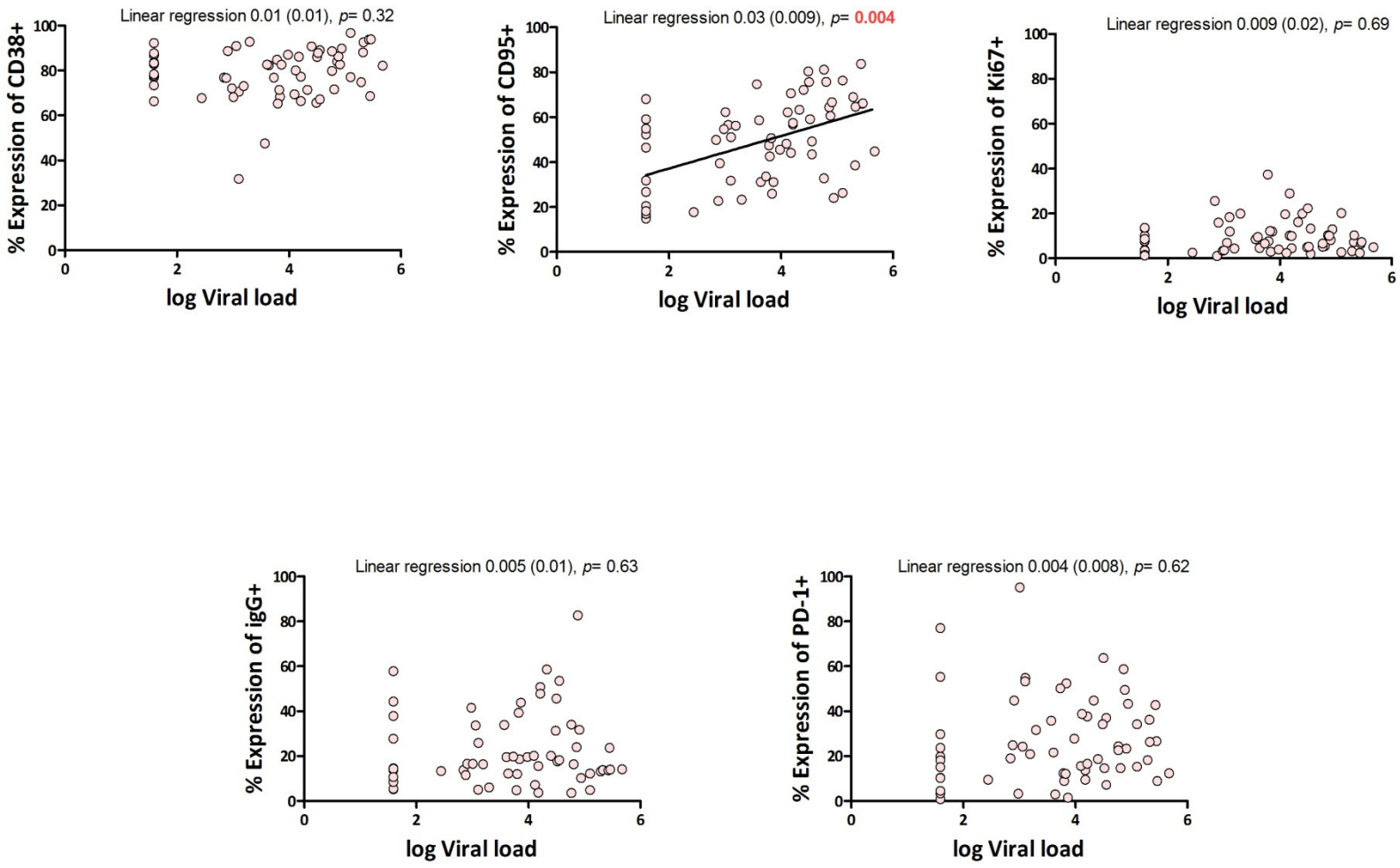

Figure 4.3.6 The relationship between markers of activation on B cells and log viral load. CD95 was the only expression marker to associate positively with log viral load. The Standard Error is shown in parenthesis.

We then went on to measure the relationship between the magnitude and number of HIV peptides targeted by CTL and the expression of any of the activation markers on $CD4^+$ T cells, $CD8^+$ T cells and B cells. First we looked at the relationship between the expression of markers of activation on $CD8^+$ T cells and the magnitude of HIV peptides targeted by CTL (see Figure 4.3.7). We did not find any significant association between the expression of any of the markers of activation on $CD8^+$ T cells and the magnitude of HIV peptides targeted by CTL.

Figure 4.3.8 shows the relationship between the magnitude of HIV peptides targeted by CTL and the expression of markers of activation on $CD4^+$ T cells. There was no significant association between the expression of any markers of activation on $CD4^+$ T cells and the magnitude of HIV peptides targeted by CTL.

We next investigated the relationship between the expression of activation markers on B cells and the magnitude of HIV peptides targeted by CTL (see Figure 4.3.9). The only marker that had a significant positive association with the magnitude of the immune response was the PD-1 marker. For every one percent increase in the expression of PD-1, there was an increase magnitude of immune responses by 21.32 units (p= 0.02).

We next investigated the relationship between the number of HIV peptides targeted by CTL and the expression of any activation markers on $CD4^+$ T cell, $CD8^+$ T cells, and B cells. Figure 4.3.10 shows the relationship between activation markers on $CD8^+$ T cells and the breadth of immune responses. There was no significant association between any activation marker on $CD8^+$ T cells and the breadth of immune responses.

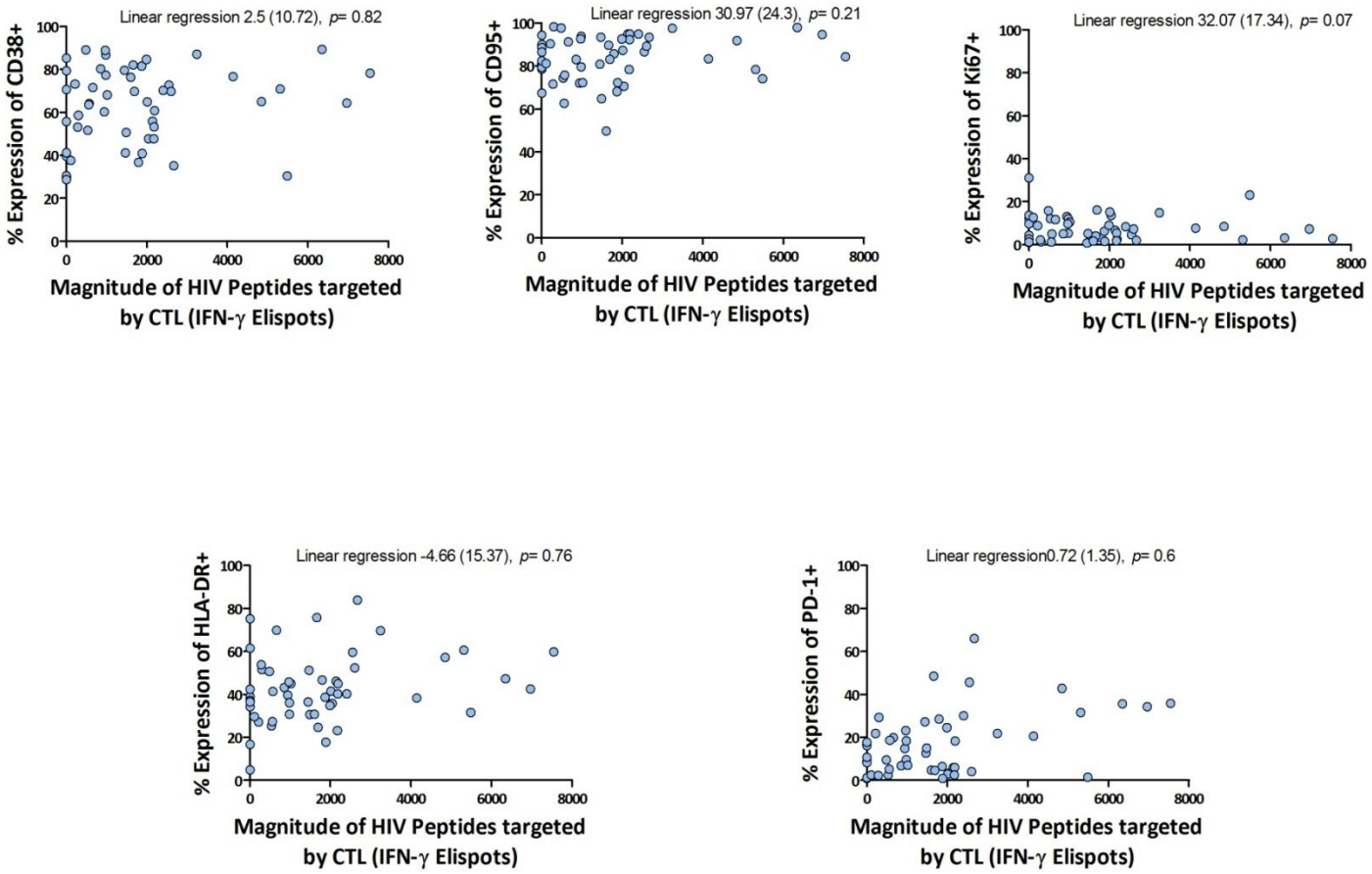

Figure 4.3.7 The relationship between markers of $CD8^+$ T cell activation and the magnitude of HIV peptides targeted by CTL. There was no significant association between the magnitude of immune responses and the expression of any activation markers on $CD8^+$ T cells. The Standard Error is shown in parenthesis.

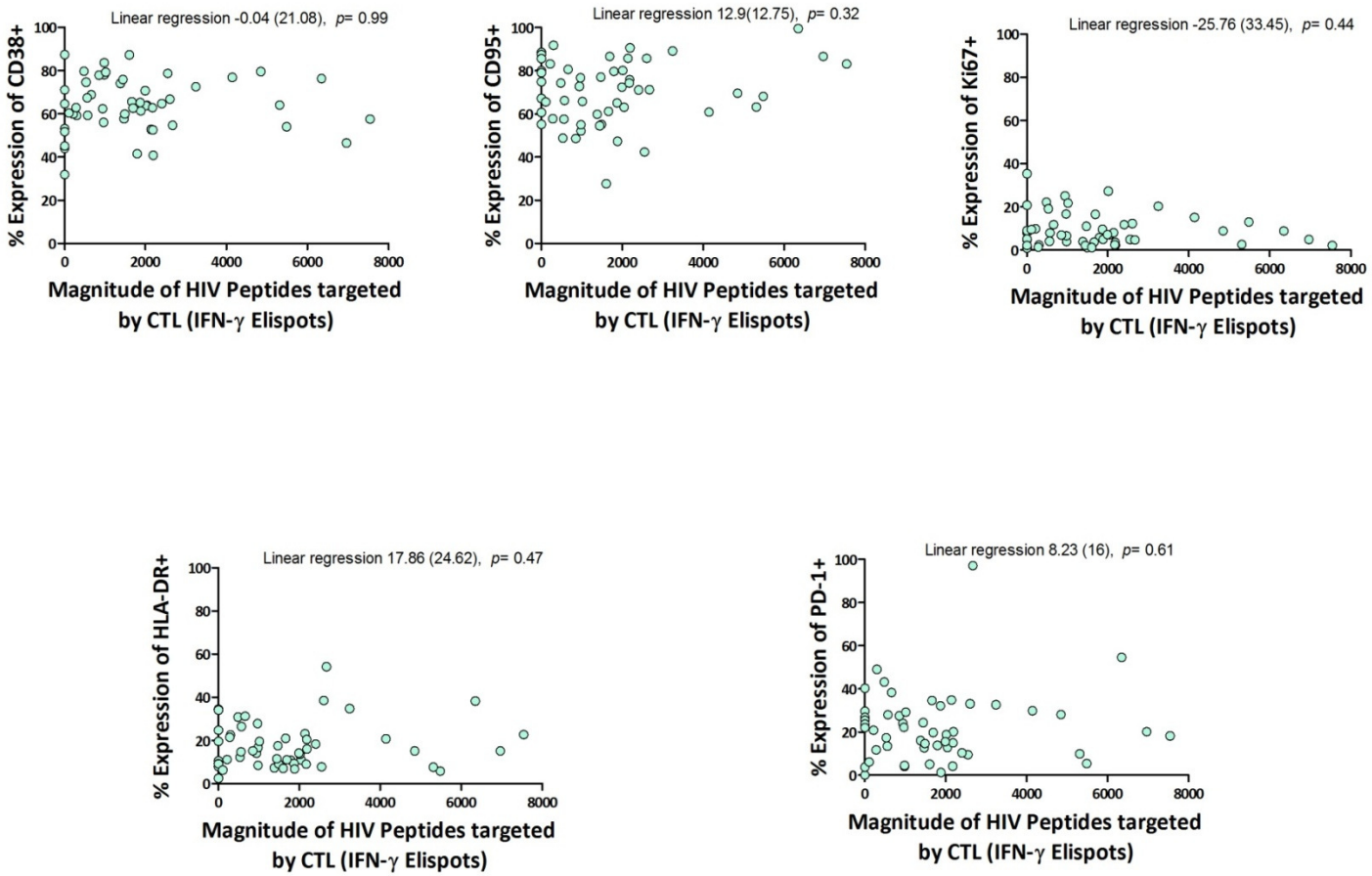

Figure 4.3.8 The relationship between the magnitude of immune responses and activation markers on $CD4^+$ T cells. There was no significant association between the expression of any activation markers on $CD4^+$ T cells and the magnitude of the immune response. The Standard Error is shown in parenthesis.

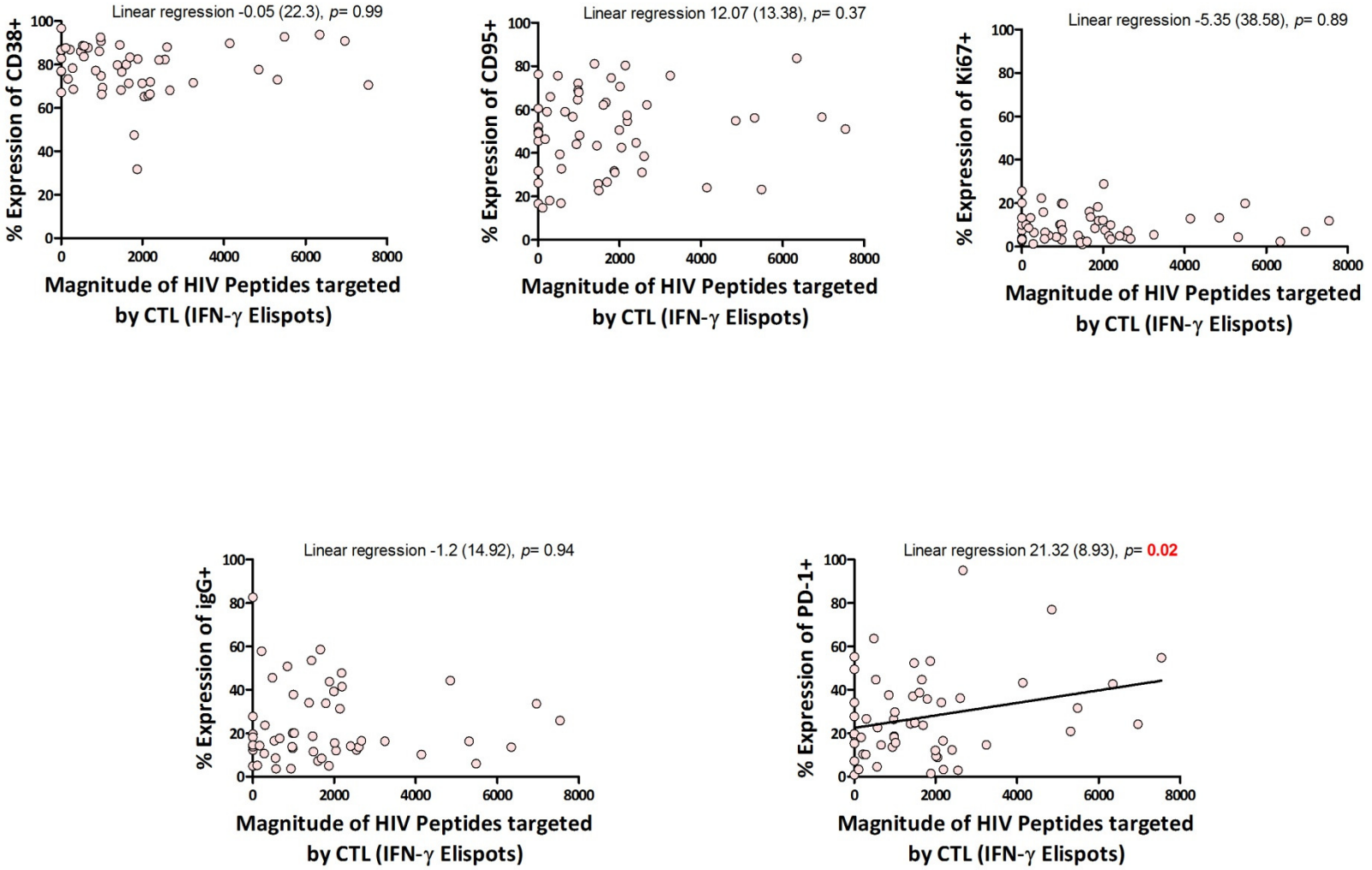

Figure 4.3.9 The relationship between activation markers on B cells and the magnitude of the immune response. PD-1 had a significant positive association with the magnitude of HIV peptides targeted by CTL. The Standard Error is shown in parenthesis.

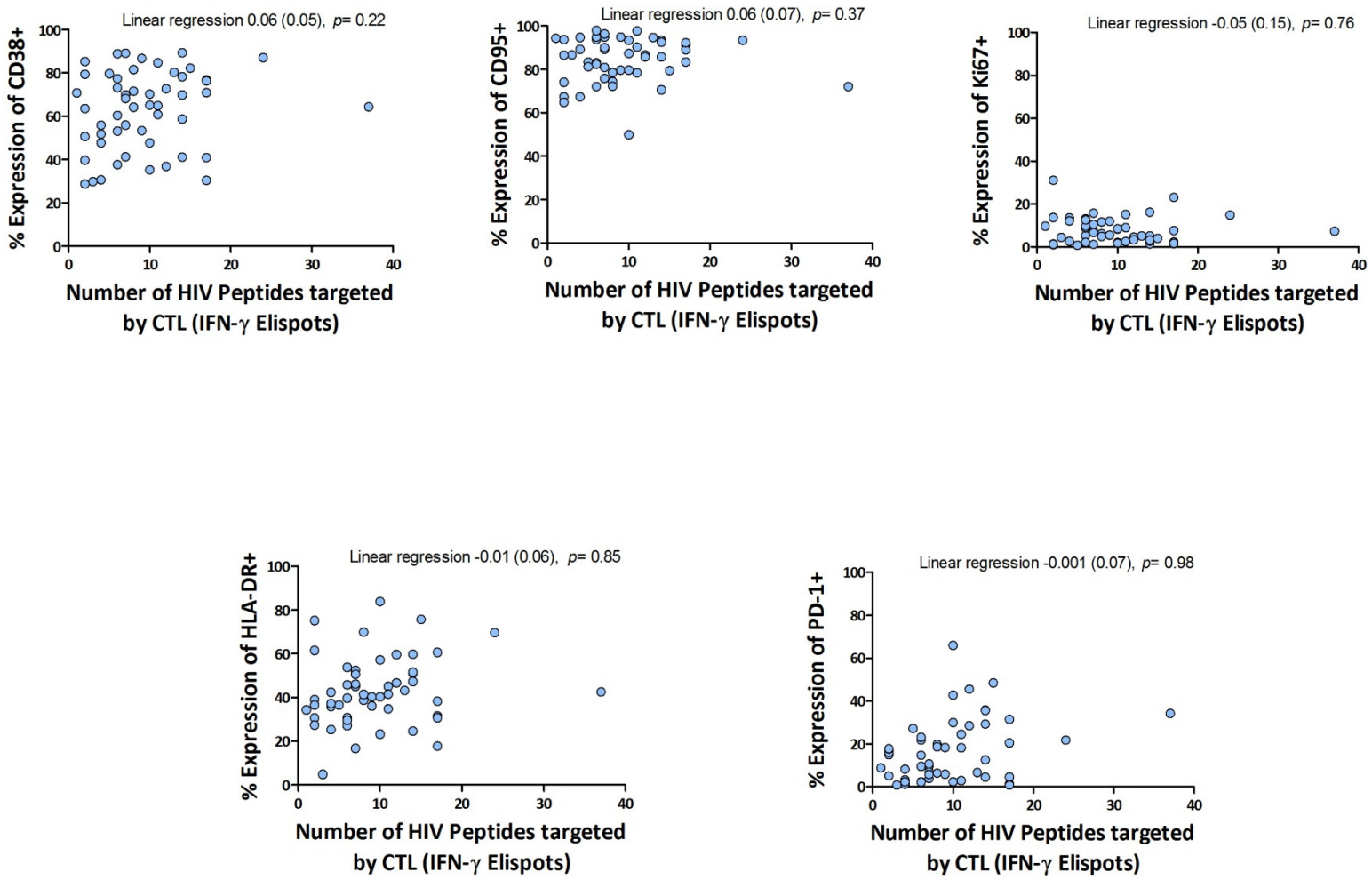

Figure 4.3.10 The relationship between activation markers on CD8$^+$ T cells and the number of HIV peptides targeted by CTL. There was no association between the expression of any activation marker on CD8$^+$ T cells and the breadth of immune responses. The Standard Error is shown in parenthesis.

Figure 4.3.11 shows the relationship between the expression of activation markers on $CD4^+$ T cells and the number of HIV peptides targeted by CTL. There was no association between the expression of any activation marker on $CD4^+$ T cells and the breadth of the immune response.

We next investigated the relationship between the expression of activation markers on B cells and the number of HIV peptides targeted by CTL (see Figure 4.3.12). There was no association between the expression of any activation marker on B cells and the breadth of the immune response.

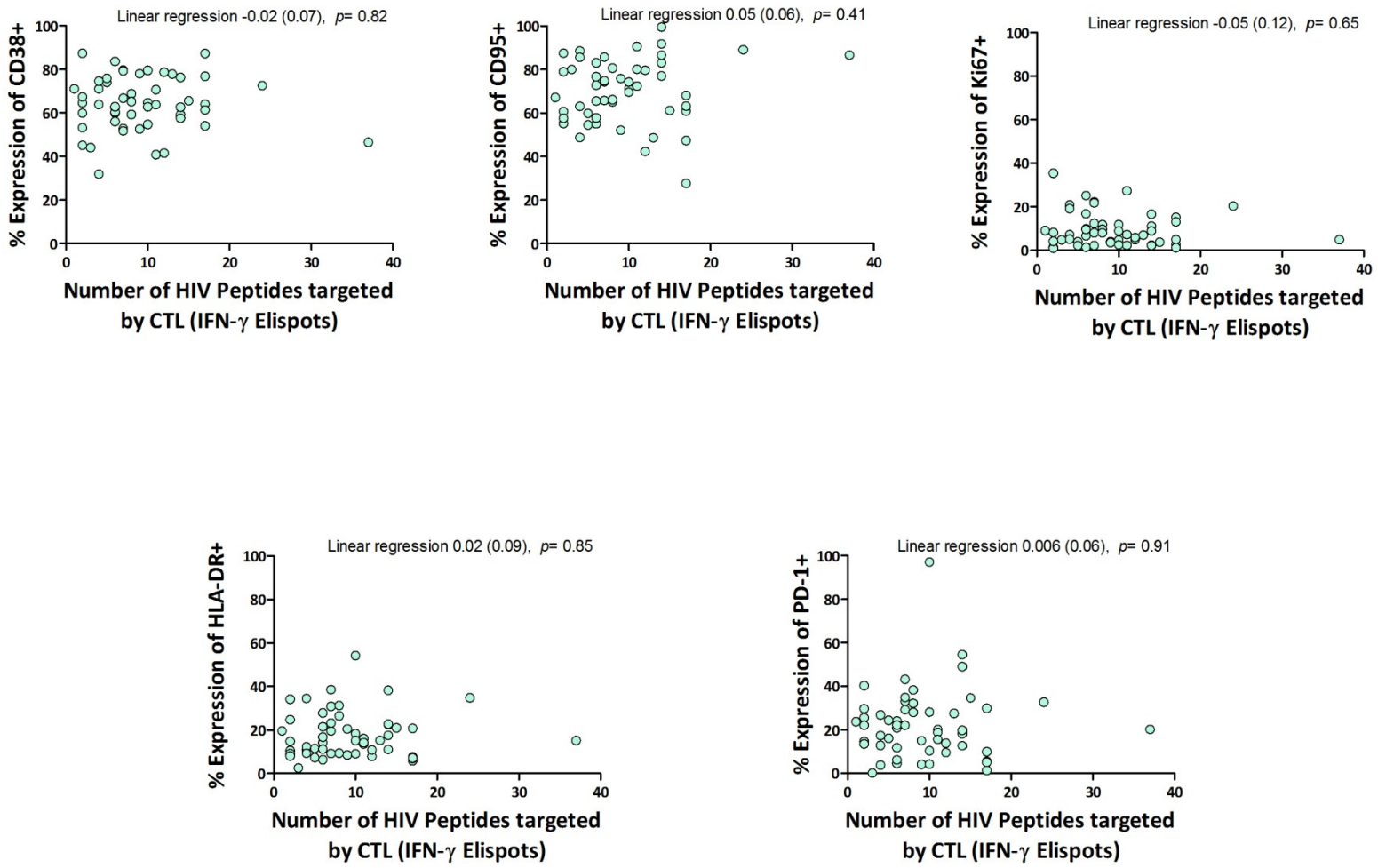

Figure 4.3.11 The relationship between activation markers on $CD4^+$ T cells and the breadth of immune responses. There was no significant association between the expression of any activation marker on $CD4^+$ T cells and the breadth of the immune response. The Standard Error is shown in parenthesis.

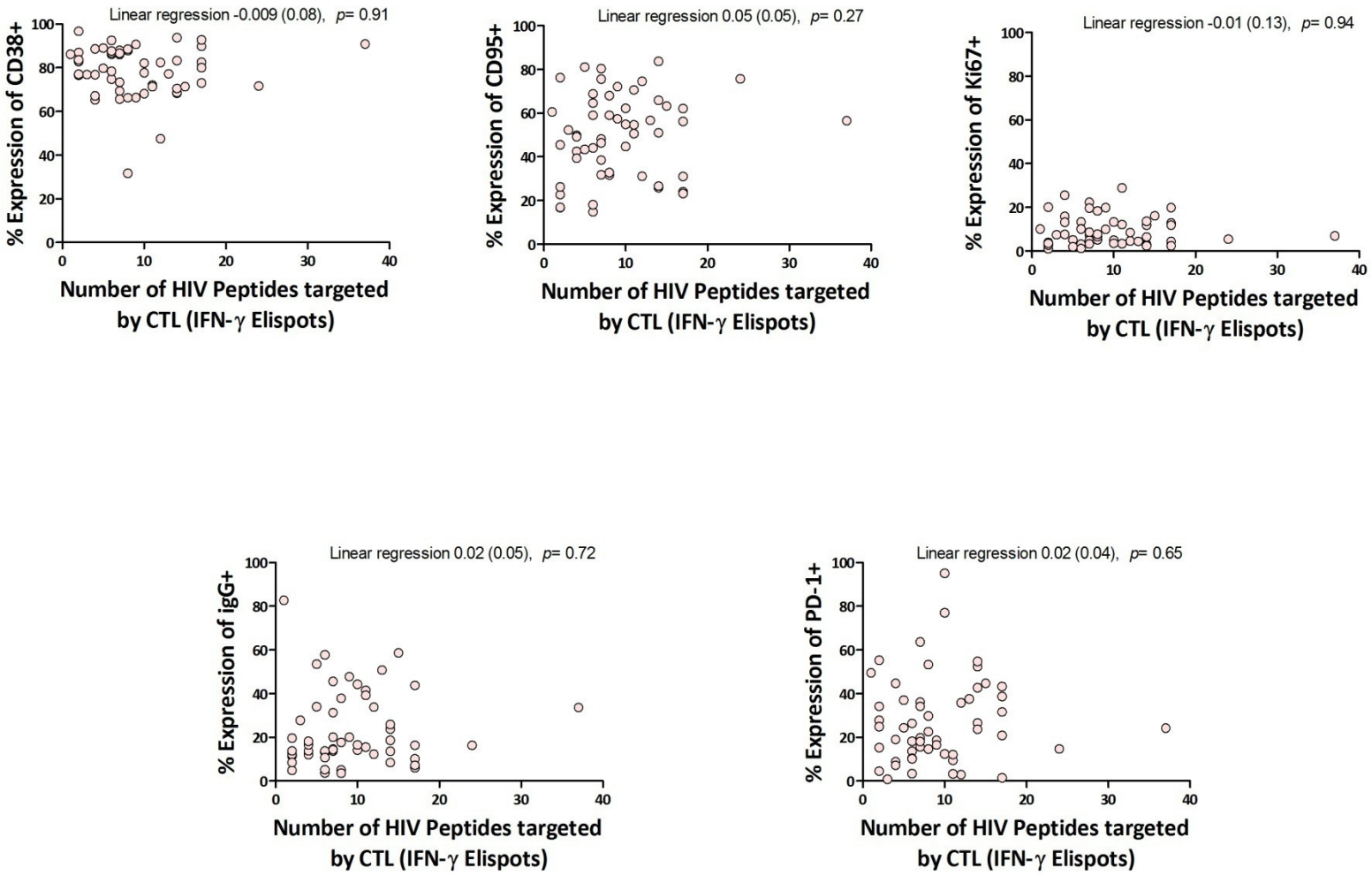

Figure 4.3.12 The relationship between activation markers on B cells and the breadth of immune responses. There was no significant association between the expression of any activation marker on B cells and the breadth of the immune response. The Standard Error is shown in parenthesis.

4.3.2 ASSOCIATION BETWEEN *IL-10* GENETIC VARIANTS AND MARKERS OF $CD4^+$, $CD8^+$ T CELLS AND B CELLS

To determine the association between *IL-10* genetic variants and markers of activation, we measured the percentage of cells positive for each marker within T and B cell subsets. For this part of the analysis we focused on 63 individuals from the Sinikithemba cohort of chronic infection.

In $CD4^+$ T cells and $CD8^+$ T cells we measured the association between *IL-10* genetic variants and the following markers of activation: CD38, CD95, Ki67, HLA-DR and PD-1. Within the B cells subset, to determine the association between IL-10 genetic variants and markers of B cell activation, we measured the following markers of B cell activation: CD38, CD95, IgG, Ki67 and PD-1.

We first investigated whether each genotype had an effect on activation, without grouping. Figure 4.3.13 shows the association between *IL-10*-592 genotype and markers of $CD4^+$ T cell activation. The -592 genotype had no significant association with CD38 or Ki67 expression on $CD4^+$ T cells (p= 0.68 and 0.23 respectively). However, we did find a significant association between -592 genotype and CD95, HLA-DR and PD-1 expression on $CD4^+$ T cells (p= 0.04, 0.01 and 0.03 respectively). With the significant association between -592 genotype and CD95 expression in $CD4^+$ T cells, we found that the -592AA group had a significantly higher median expression of CD95 as compared to the -592CC or -592CA groups (p= 0.03 and 0.02 respectively); the -592AA genotype had a significantly higher median expression of HLA-DR as compared to the -592CC or -592CA (p= 0.03 and 0.005 respectively); and the -592AA

group had a significantly higher median expression of PD-1 compared to the -592CA group (p= 0.01).

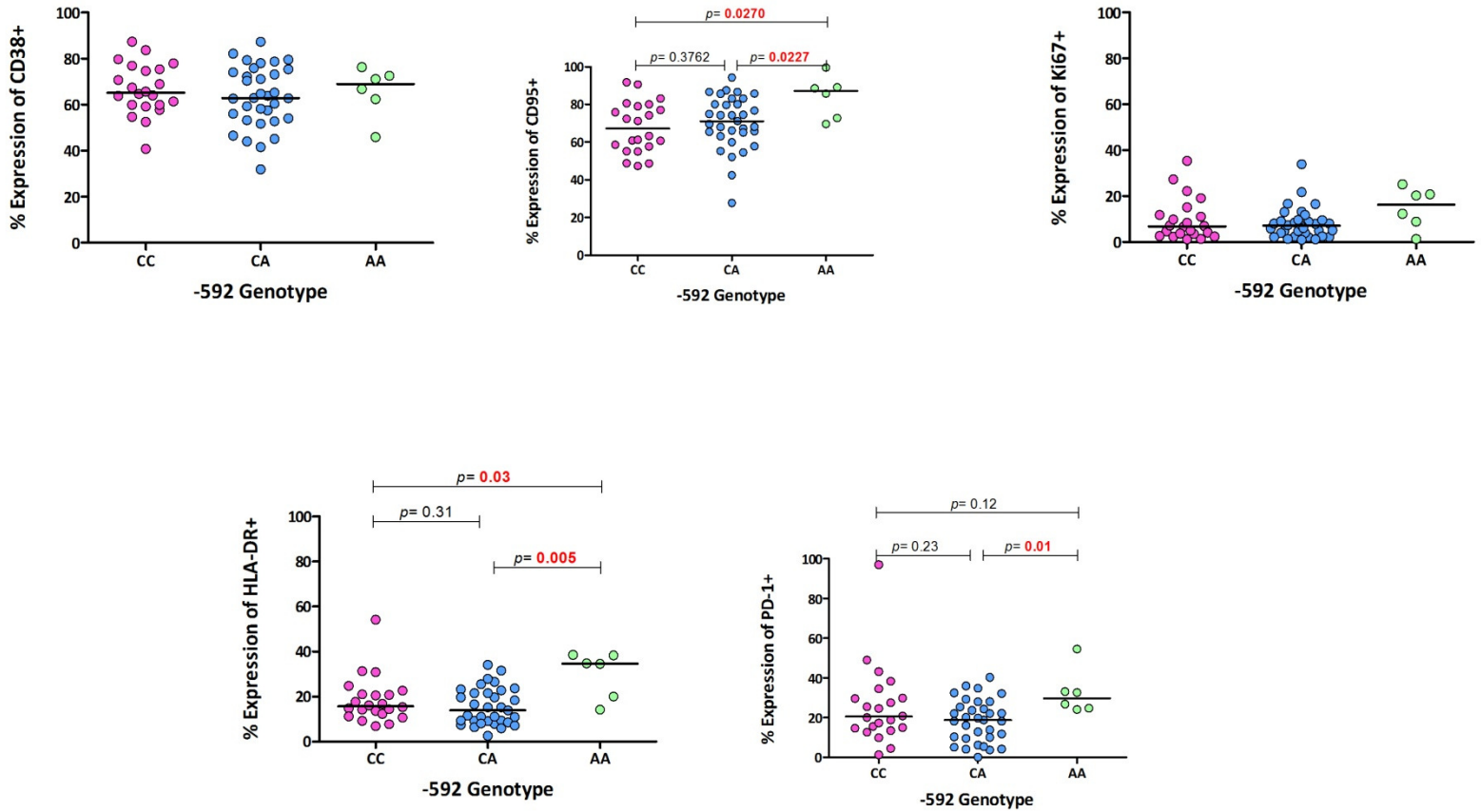

Figure 4.3.13 Association between -592 genotype and markers of CD4$^+$ T cell activation. The association between -592 genotype and CD4$^+$ T cell activation was measured by the expression of CD38, CD95, HLA-DR, Ki67 and PD-1. -592 genotype did not associate with CD38 or Ki67, but did significantly associate with CD95, HLA-DR and PD-1 expression.

We next investigated the association between *IL-10*-1082 genotype and markers of $CD4^+$ T cell activation (see Figure 4.3.14). This was performed by measuring the expression of CD38, CD95, Ki67, HLA-DR and PD-1. The -1082 genotype did not associate with any of the markers expressed on $CD4^+$ T cells. Here, we found that the -1082 genotype did not significantly associate with CD38, CD95, Ki67, HLA-DR or PD-1 (p= 0.54, 0.15, 0.39, 0.27 and 0.62 respectively).

We then investigated the association between the -592 genotype and markers of activation on $CD8^+$ T cells. We measured activation by measuring the expression of the following markers: CD38, CD95, Ki67, HLA-DR and PD-1. Figure 4.3.15 shows the association between *IL-10*-592 genotype and these markers of $CD8^+$ T cell activation. We did not find any significant association between the -592 genotype and any markers of activation on $CD8^+$ T cells. The -592 genotype did not associate with CD38, CD95, Ki67, HLA-DR or PD-1 (p= 0.45, 0.51, 0.66, 0.2 and 0.82 respectively).

To determine the association between the *IL-10*-1082 genotype and activation of $CD8^+$ T cells, we measured the following markers of activation: CD38, CD95, Ki67, HLA-DR and PD-1 (see Figure 4.3.16). The -1082 genotype did not significantly associate with the expression of CD38, CD95, Ki67 and PD-1 (p= 0.73, 0.11, 0.27 and 0.5 respectively). However, we found a significant association between the -1082 genotype and the expression of HLA-DR on $CD8^+$ T cells (p= 0.05). Here we found that the -1082AA group had a significantly higher median expression of HLA-DR than the -1082AG group (p= 0.02).

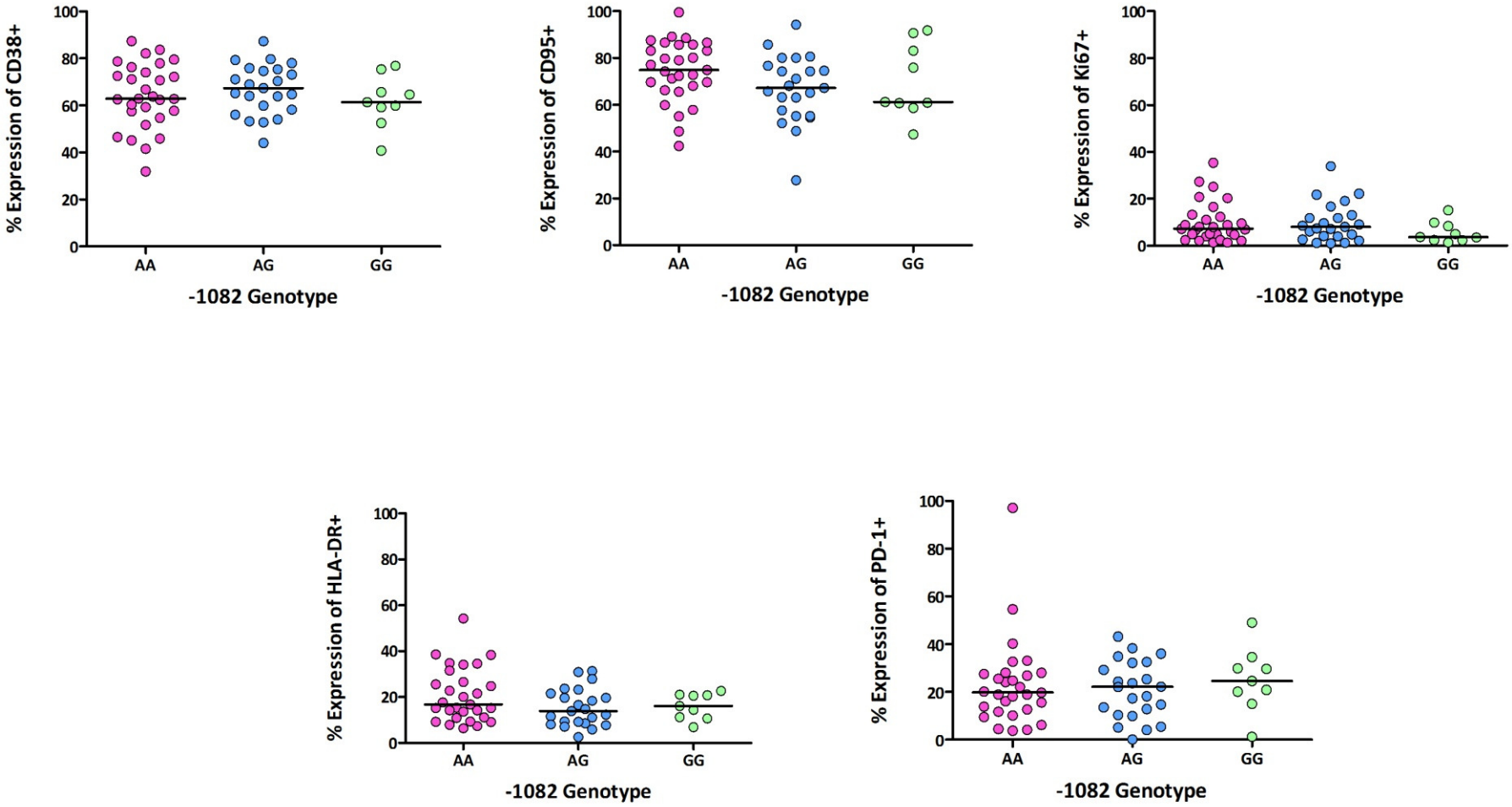

Figure 4.3.14 Association between the -1082 genotype and markers of activation on CD4$^+$ T cells. The -1082 genotype did not significantly associate with any of the markers of CD4$^+$ T cell activation.

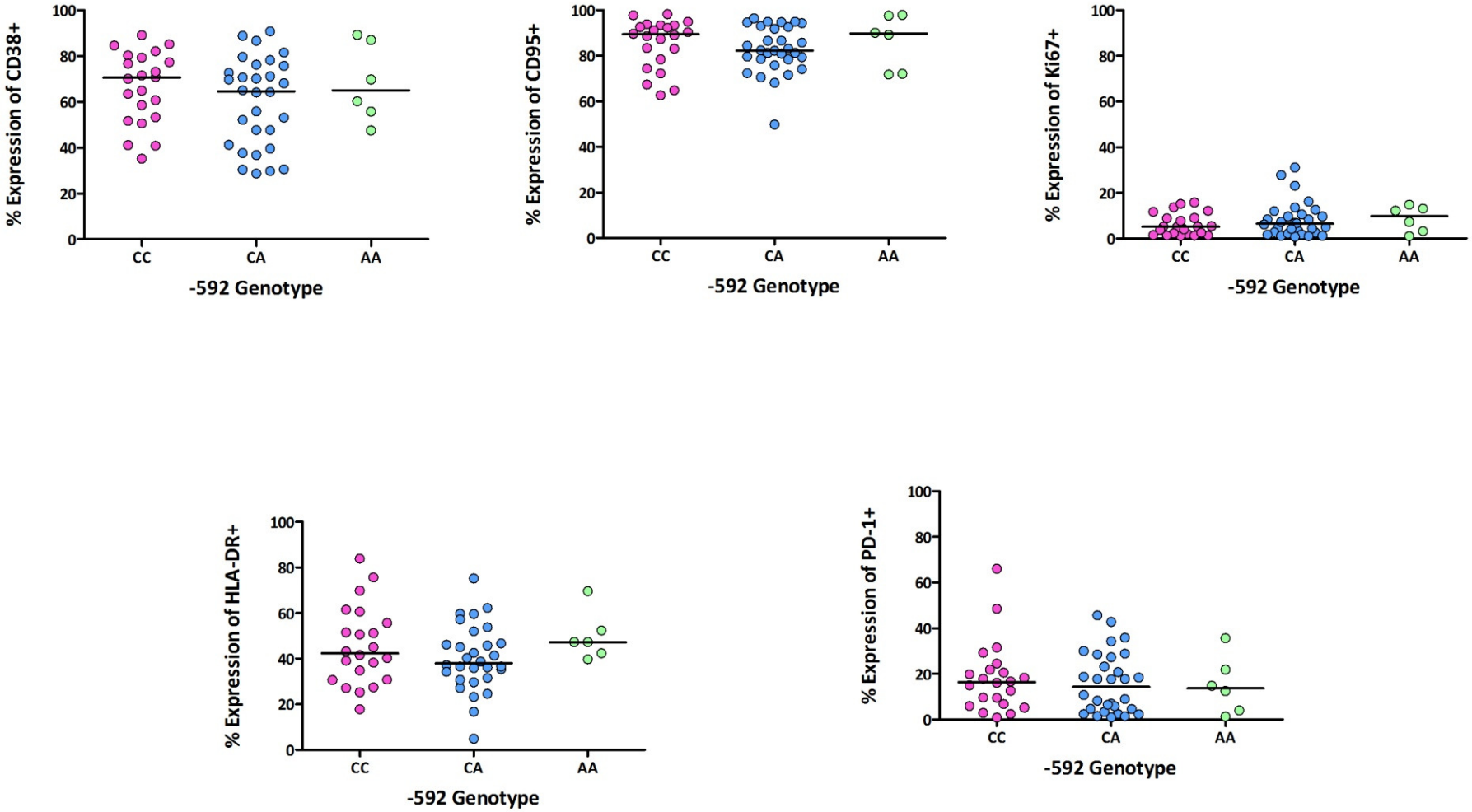

Figure 4.3.15 Association between -592 genotype and markers of activation on CD8$^+$ T cells. The -592 genotype did not significantly associate with any markers of activation on CD8$^+$ T cells.

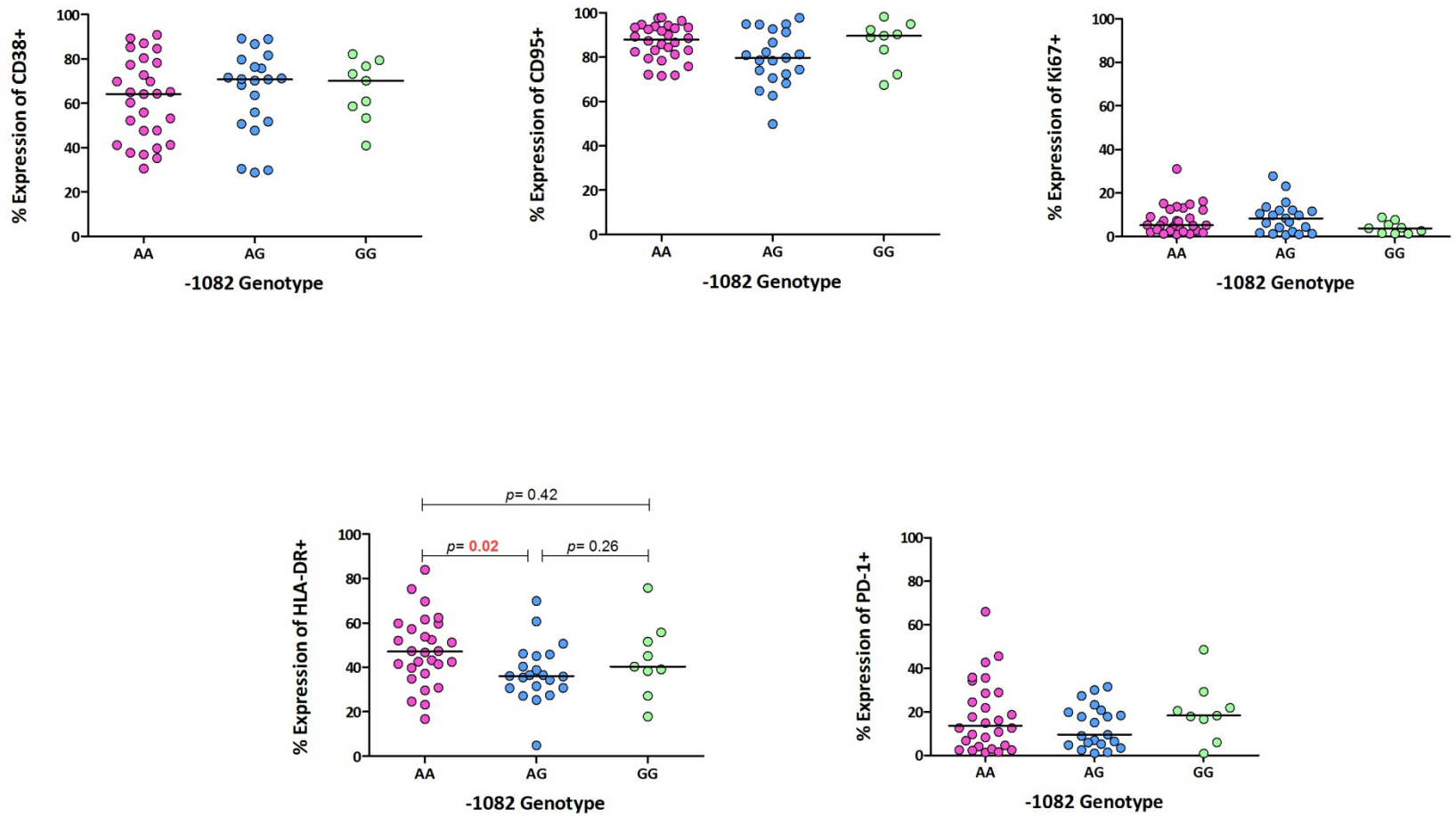

Figure 4.3.16 The association between -1082 genotype and markers of activation on CD8$^+$ T cells. The -1082 genotype showed a significant association with the expression of HLA-DR (p= 0.047).

We next investigated the association between *IL-10*-592 genotype and markers of activation of B cells. We measured the following markers of activation: CD38, CD95, Ki67, IgG and PD-1. We first looked at the association between the -592 genotype and these markers of activation (see Figure 4.3.17). We found no significant association between the -592 genotype and the expression of CD38, CD95, Ki67 and PD-1 (p= 0.78, 0.83, 0.86 and 0.86 respectively). However, we found a trend between the -592 genotype and expression of IgG (p= 0.07). We found that the -592CC genotype had a significantly higher median of IgG expression compared to the -592AA group (p= 0.03).

We next looked at the association between -1082 genotype and markers of activation of B cells, by looking at the following markers: CD38, CD95, Ki67, IgG and PD-1 (see Figure 4.3.18). We did not find any significant association between the -1082 genotype and the expression of CD38, CD95, Ki67 and PD-1 (*p*= 0.79, 0.92, 0.54 and 0.55 respectively). However, we found a significant association between the -1082 genotype and the expression of IgG on B cells (*p*= 0.02). Here we found that the -1082GG group had a significantly higher median expression of IgG than the -1082AA and -1082AG groups (*p*= 0.005 and 0.04 respectively).

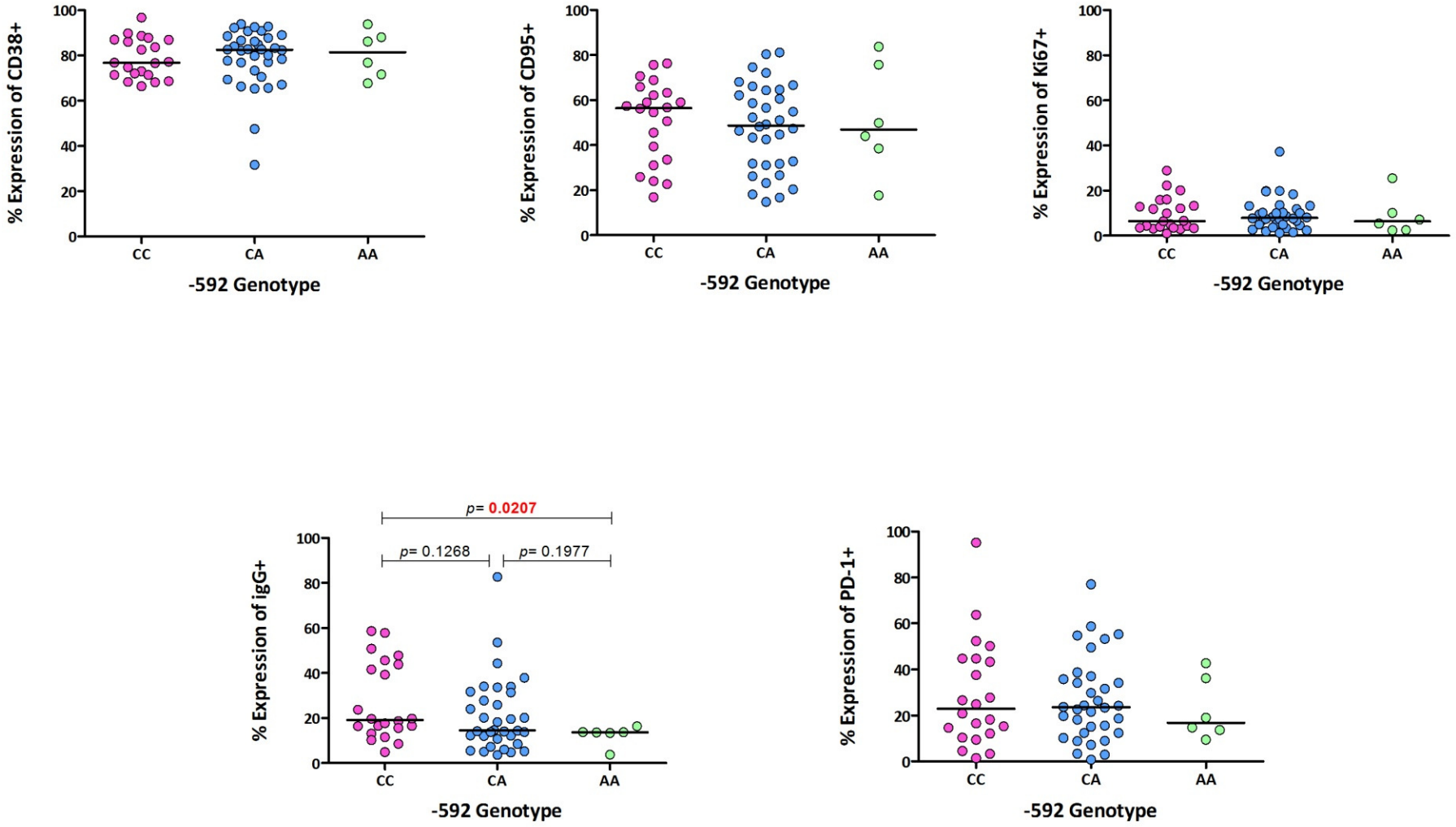

Figure 4.3.17 Association between the -592 genotype and markers of activation on B cells. The -592 genotype was significantly associated with the expression of IgG (p= 0.03).

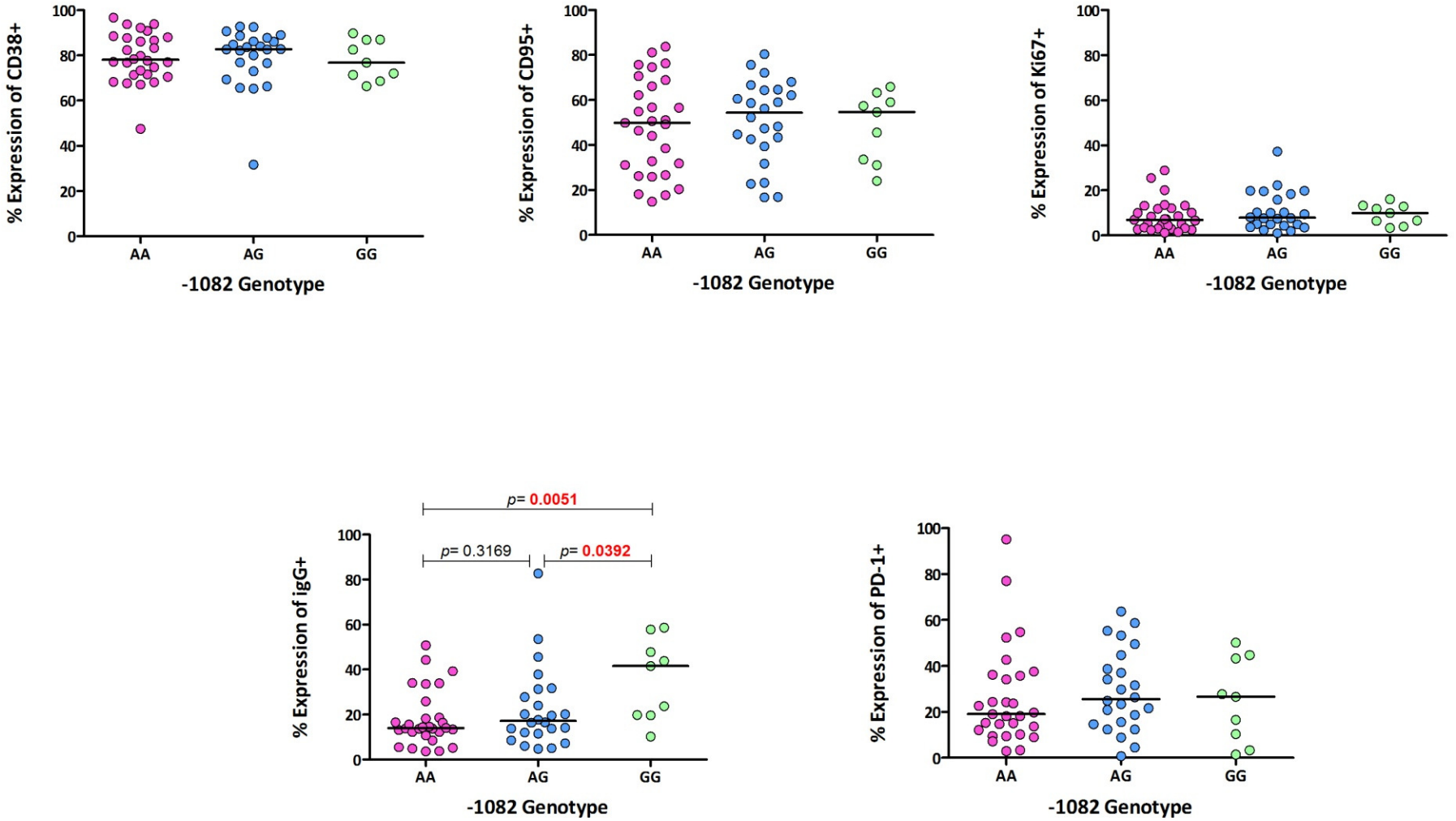

Figure 4.3.18 The association between the -1082 genotype and markers of activation on B cells. The -592 genotype was significantly associated with the expression of IgG (*p*= 0.02).

4.3.3 ASSOCIATION BETWEEN *IL-10* GENETIC VARIANTS AND CD4$^+$ T CELL PROLIFERATION AFTER IL-10 BLOCKADE

To determine the association between the role of *IL-10* genetic variants on CD4$^+$ T cell proliferation after IL-10 receptor blockade, CFSE assays were used. Carboxyfluorescein diacetate succinimidyl ester (CFSE) is used to measure cell proliferation by flow cytometry. CFSE passively diffuses into cells and combines with cellular proteins. Therefore, during cell division the CFSE is equally distributed between the two resulting cells resulting in a decrease in the percent of CFSE within cells. Therefore, the % of CFSE low cells, as measured through the gating strategy used for flow cytometry, indicates the amount of resulting proliferation.

For this part of the analysis, we focused on 40 individuals from the HPP Sinikithemba cohort. We first investigated the overall proliferation of CD4$^+$ T cells before and after IL-10 receptor blockade with anti-IL-10Rα antibody (see Figure 4.3.19). Although there was a trend towards increased proliferation following IL-10 receptor blockade, this was not significant (p= 0.09).

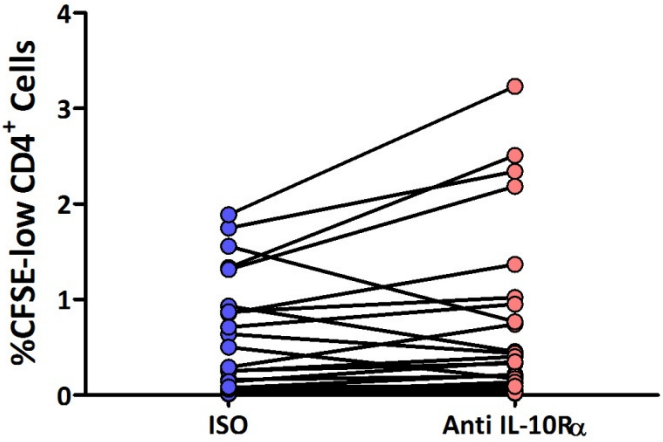

Figure 4.3.19 CD4$^+$ T cell proliferation after IL-10 receptor blockade. There appears to be a trend to increased proliferation after IL-10 receptor blockade, but this was not significant (p= 0.09).

We then went on to assess if *IL-10* genotype played a role in $CD4^+$ T cell proliferation after IL-10 receptor blockade (see Figure 4.3.20). As the samples size was small, we grouped individuals according to genotypes at both positions, placing them into extreme genotype groups, i.e. -592AA/-1082AA (low-IL-10-producing) and -592CC/-1082GG (high-IL-10-producing). We did not find any significant association between extreme *IL-10* genotype and $CD4^+$ T cell proliferation after IL-10 receptor blockade (p= 0.60).

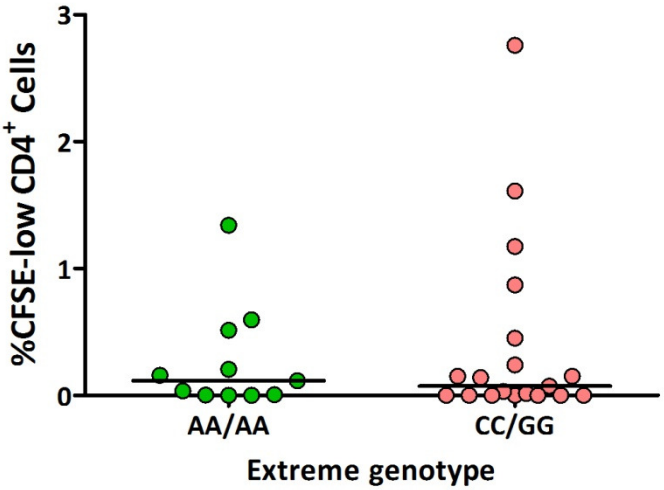

Figure 4.3.20 Association between extreme *IL-10* genotypes and $CD4^+$ T cell proliferation. There was no significant association between genotype and proliferation (p= 0.60).

We also investigated whether the viral load of the individuals played a role in $CD4^+$ T cell proliferation after IL-10 receptor blockade (see Figure 4.3.21). Here, we grouped the individuals by log viral load into a low log viral load (less than or equal to the median log viral load of 4.69 log copies/ml) or high log viral load (greater than the median log viral load of

4.69 copies/ml). Viral load did not significantly associate with $CD4^+$ T cell proliferation after IL-10 receptor blockade (p= 0.1239).

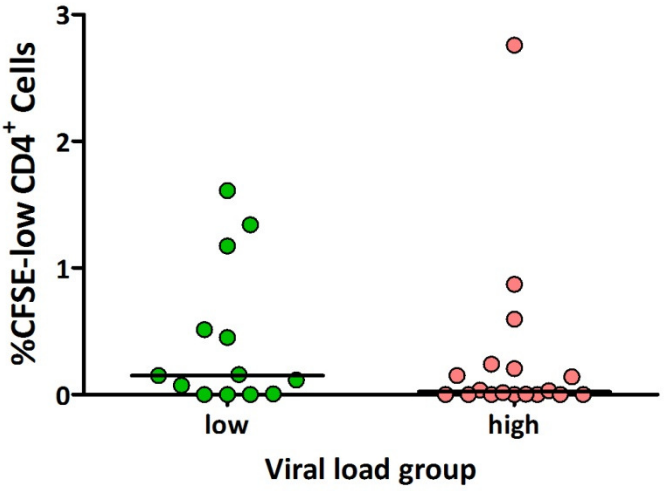

Figure 4.3.21 The association between viral load and $CD4^+$ T cell proliferation after IL-10 receptor blockade. There was no significant association between viral load and proliferation (p= 0.1239).

We next investigated whether *IL-10* haplotype had any association with proliferation after IL-10 blockade (see Table 4.3.1). We were able to analyse data for 3 of the 7 haplotypes, as we did not have sufficient numbers for the other haplotypes to make statistical comparisons. Using the Wilcoxon rank sums test, we found no significant association between any *IL-10* haplotype and proliferation after IL-10 blockade.

Table 4.3.1 Association between haplotype and proliferation after IL-10 blockade

Haplotype		n	Median IL-10Ra – ISO (IQR)	*p*-value
AAT	No	12	0.20 (0.11 - 1.02)	0.46
	Yes	9	0.16 (0.04 - 0.51)	
CGA	No	10	0.18 (0.04 - 0.60)	0.81
	Yes	11	0.15 (0.07 - 0.87)	
CGT	No	13	0.16 (0.12 - 0.51)	0.69
	Yes	8	0.20 (0.05 - 1.39)	

4.3.4 ASSOCIATION BETWEEN *IL-10* GENETIC VARIANTS AND SECRETED CYTOKINE PROFILES AFTER IL-10 BLOCKADE

To determine if IL-10 receptor blockade had an effect on the production of select cytokines, supernatants were collected and cytokine concentrations were measured for IFN-γ, IL-2, IL-6, IL-10, IL-13 and TNF-α. We first investigated whether IL-10 receptor blockade had an effect on cytokine production (see Figure 4.3.22). Overall we found that there was no significant association between IL-10 receptor blockade and levels of IFN-γ, IL-6, IL-10, IL-13 and TNF-α (p= 0.49, 0.67, 0.87, 0.32 and 0.51 respectively). However, we did find that there was a significant association between IL-10 receptor blockade and IL-2 production (p= 0.004). Figure 4.3.23 shows that the median level of IL-2 was significantly increased after IL-10 receptor blockade.

We next wanted to investigate if extreme *IL-10* genotype influenced the expression of these cytokines after IL-10 receptor blockade (see Table 4.3.2). We found that within individuals with the -592AA/-1082AA genotype there was a significant increase in IL-2 production after IL-10 receptor blockade (p= 0.02).

We then wanted to determine whether viral load played a role in cytokine production after IL-10 receptor blockade (see Table 4.3.3). We found that within individuals in the high log viral load group, there was a significant increase in IL-2 production after IL-10 receptor blockade (p= 0.008).

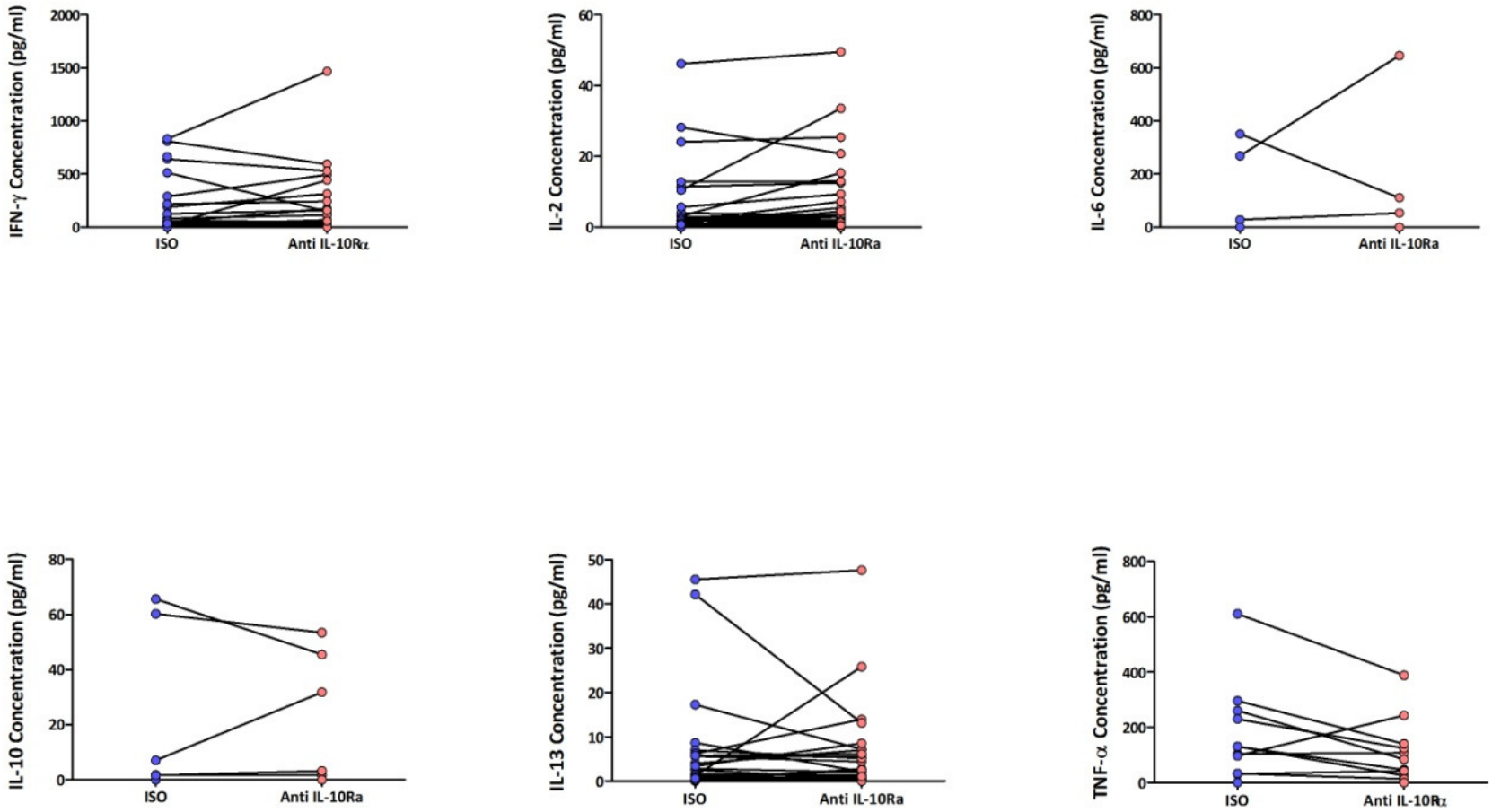

Figure 4.3.22 Effect of IL-10 receptor blockade on cytokine production. IL-10 receptor blockade did not significantly affect the production of IFN-γ, IL-6, IL-10, IL-13 or TNF-α. IL-2 production significantly increased following IL-10 receptor blockade (p= 0.004).

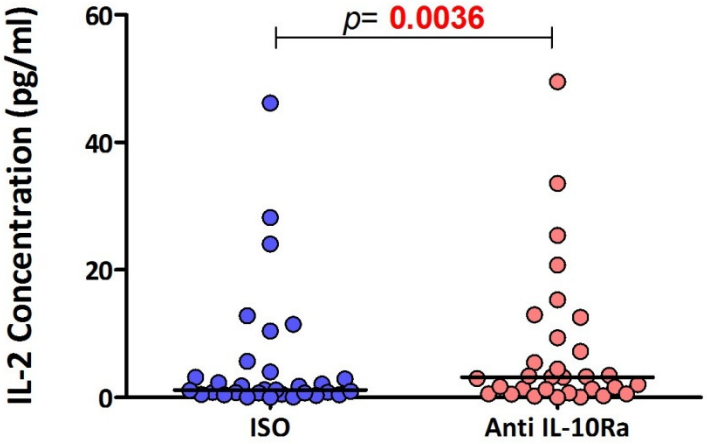

Figure 4.3.23 Median IL-2 levels are increased after IL-10 receptor blockade.

Table 4.3.2 Association of extreme *IL-10* genotype on cytokine production after IL-10 receptor blockade

Cytokine	-592 AA or -1082 AA			-592 CC or -1082 GG		
	n	Median difference IL10Ra – ISO (IQR)	*p*-value	n	Median difference IL10Ra – ISO (IQR)	*p*-value
IFN-γ	14	9.923 (-215.485 – 43.475)	0.95	25	0.000 (-11.280 – 31.635)	0.52
TNF-α	10	-11.600 (-83.440 – 361.505)	0.85	21	-3.140 (-96.200 – 11.680)	0.35
IL-2	15	1.140 (0.230 – 4.25)	**0.02**	25	0.180 (-0.035 – 0.550)	0.08
IL-6	7	131.065 (-19.025 – 377.480)	0.58	16	-5.545 (-49.550 – 34.463)	0.85
IL-10	15	5.930 (-25.905 – 79.560)	0.42	24	-2.390 (-75.515 – 14.713)	0.34
IL-13	15	0.800 (-0.775 – 2.530)	0.42	25	-0.070 (-0.425 – 0.510)	0.67

Table 4.3.3 Association between viral load on cytokine production after IL-10 receptor blockade

Cytokine	Low viral load*			High viral load*		
	n	Median difference IL10Ra – ISO (IQR)	*p*-value	n	Median difference IL10Ra – ISO (IQR)	*p*-value
IFN-γ	15	22.005 (-16.755 – 202.76)	0.27	17	0.185 (-7.785 – 34.185)	0.64
TNF-α	13	0.000 (-83.350 – 361.505)	0.57	11	-3.140 (-155.305 – 16.330)	0.46
IL-2	16	1.078 (-0.118 – 3.988)	0.06	17	0.530 (0.180 – 1.340)	**0.008**
IL-6	9	0.000 (-22.705 – 131.065)	0.46	8	-5.545 (-310.310 – 203.173)	0.74
IL-10	15	14.755 (-25.905 – 107.125)	0.22	17	-0.145 (-20.120 – 5.930)	0.75
IL-13	16	0.540 (-1.295 – 3.780)	0.39	17	0.390 (-0.185 – 1.035)	0.18

* Viral load was classified as being low and high, based on the median for the group, which was 4.69 log copies/ml.

We investigated whether *IL-10* haplotype had an association with cytokine expression after IL-10 blockade. We were able to analyse only three of the six cytokines measured, as the other cytokines did not have adequate samples size. The three cytokines analysed were IL-2, IL-13 and IFN- γ. Table 4.3.4 shows the association between *IL-10* haplotype and IL-2 expression after IL-10 blockade. Using the Wilcoxon rank sums test we found that there was a significant association between *IL-10* haplotype and IL-2 expression after IL-10 blockade. Individuals with the AAT haplotype had significantly higher IL-2 levels than those without, following IL-10R blockade (median 1.34 vs. 0.47pg/ml, p=0.02). Also, individuals with the CGT haplotype had significantly lower IL-2 levels than those without (median 0.16 vs. 1.11, p= 0.03).

Table 4.3.4 Association between *IL-10* haplotype and IL-2 expression after IL-10 receptor blockade

Haplotype		n	Median IL-10Ra – ISO (IQR)	*p*-value
AAT	No	16	0.47 (0.14 - 1.67)	**0.02**
	Yes	11	1.34 (0.80 - 4.25)	
CGA	No	12	1.24 (0.67 - 3.79)	0.09
	Yes	15	0.49 (0.16 - 2.26)	
CGT	No	20	1.11 (0.54 - 3.53)	**0.03**
	Yes	7	0.16 (0.13 - 0.49)	

Table 4.3.5 shows the association between the *IL-10* haplotype and IL-13 expression after IL-10 blockade. We found no significant association between any haplotype and IL-13 expression after IL-10 blockade.

Table 4.3.5 Association between *IL-10* haplotype and IL-13 expression after IL-10 blockade

Haplotype		n	Median IL-10Ra – ISO (IQR)	*p*-value
AAT	No	7	2.49 (0.43 - 7.72)	0.4
	Yes	4	1.55 (0.71 - 2.52)	
CGA	No	4	1.55 (0.71 - 2.52)	0.4
	Yes	7	2.49 (0.43 - 7.72)	
CGT	No	8	2.16 (0.73 - 2.73)	0.48
	Yes	3	7.72 (0.20 - 24.62)	

Table 4.3.6 shows the association between *IL-10* haplotype and IFN- γ expression after IL-10 pathway blockade. We found no significant association between any *IL-10* haplotype and IFN-γ expression after IL-10 receptor blockade.

Table 4.3.6 Association between IL-10 haplotype and IFN- γ expression after IL-10 receptor blockade

Haplotype		n	Median IL-10Ra – ISO (IQR)	*p*-value
AAT	No	11	31.63 (4.12 - 202.77)	0.97
	Yes	8	35.18 (20.68 - 86.12)	
CGA	No	8	35.18 (20.68 - 86.12)	0.97
	Yes	11	31.63 (4.12 - 202.77)	
CGT	No	14	29.26 (22.01 - 123.21)	0.96
	Yes	5	34.19 (4.12 - 43.17)	

4.3.5 PLASMA CYTOKINE PROFILES AT MATCHED TIME POINT

We measured select cytokines in plasma samples from matched time points to the PBMCs used in the IL-10 receptor blockade assays. Here we wanted to investigate whether the proliferation profiles, had an effect on cytokine expression. We measured IFN-γ, IL-2, IL-6, IL-10, IL-13 and TNF-α in 40 individuals from the HPP Sinikithemba cohort (see Table 4.3.7). We measured overall cytokine expression, as well as whether extreme *IL-10* genotypes associated with cytokine production. Overall the plasma taken from individuals at different timepoints did not associate with extreme *IL-10* genotype combinations.

We also investigated the association between cytokine expression and *IL-10* haplotype in plasma samples matched to timepoint of PBMCs used in the blockade assays. This was to determine the association between IL-10 levels at the same timepoint of the PBMCs used to carry out the proliferation assay following IL-10 blockade. With regards to the other cytokines measured, we found no significant association between any *IL-10* haplotype and any of the cytokines measured, i.e. IL-10 (Table 4.3.8), IL-2 (Table 4.3.9), IL-6 (Table 4.3.10), IL-13 (Table 4.3.11), IFN- γ (Table 4.3.12) and TNF-α (Table 4.3.13).

Table 4.3.7 Association between plasma cytokine measurement and extreme *IL-10* genotype combination

Cytokine	Overall		-592AA or -1082AA		-592CC or -1082GG		*p*-value
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	
IFN-γ	37	11.83 (8.04 – 18.35)	14	12.06 (8.48 – 17.68)	23	11.83 (6.89 – 23.40)	0.94
TNF-α	39	14.86 (13.42 – 22.49)	14	14.46 (13.72 – 17.08)	25	15.65 (13.42 – 22.92)	0.46
IL-2	40	5.25 (3.34 – 8.51)	15	5.13 (3.41 – 8.56)	25	5.39 (3.12 – 8.46)	0.93
IL-6	40	5.24 (3.76 – 7.98)	15	4.83 (3.16 – 8.18)	25	5.27 (4.84 – 7.78)	0.43
IL-10	39	31.57 (22.88 – 42.14)	15	33.31 (21.33 – 45.17)	24	29.06 (23.55 – 41.85)	0.82
IL-13	35	2.93 (1.38 – 4.31)	13	2.93 (1.36 – 4.31)	22	2.73 (1.65 – 4.17)	0.72

Table 4.3.8 Association between *IL-10* haplotype and plasma IL-10 expression

Haplotype		n	Median plasma IL-10 (IQR)	*p*-value
AAT	No	24	29.06 (23.55 - 41.85)	0.8174
	Yes	15	33.31 (21.33 - 45.17)	
CGA	No	17	31.57 (22.88 - 40.76)	0.8986
	Yes	22	30.84 (24.22 - 42.14)	
CGT	No	25	32.05 (22.88 - 42.14)	0.8836
	Yes	14	28.26 (22.88 - 41.57)	

Table 4.3.9 Association between *IL-10* haplotype and plasma IL-2 expression

Haplotype		n	Median plasma IL-2 (IQR)	*p*-value
AAT	No	25	5.39 (3.12 - 8.46)	0.9332
	Yes	15	5.13 (3.41 - 8.56)	
CGA	No	17	5.13 (3.42 - 8.56)	0.7844
	Yes	23	5.39 (3.08 - 8.46)	
CGT	No	26	5.19 (3.41 - 6.72)	0.6399
	Yes	14	6.16 (3.12 - 9.74)	

Table 4.3.10 Association between *IL-10* haplotype and plasma IL-6 expression

Haplotype		n	Median plasma IL-6 (IQR)	*p*-value
AAT	No	25	5.27 (4.84 - 7.78)	0.4341
	Yes	15	4.83 (3.16 - 8.18)	
CGA	No	17	4.83 (3.56 - 7.78)	0.4601
	Yes	23	5.27 (4.84 - 8.32)	
CGT	No	26	5.15 (3.56 - 8.18)	0.7230
	Yes	14	5.49 (4.02 - 7.78)	

Table 4.3.11 The association between *IL-10* haplotype and plasma IL-13 expression

Haplotype		n	Median plasma IL-13 (IQR)	*p*-value
AAT	No	22	2.73 (1.65 - 4.17)	0.7200
	Yes	13	2.93 (1.36 - 4.31)	
CGA	No	15	2.93 (1.36 - 4.31)	0.7015
	Yes	20	2.73 (1.51 - 4.91)	
CGT	No	22	3.03 (1.36 - 4.85)	0.9049
	Yes	13	2.07 (1.93 - 3.88)	

Table 4.3.12 Association between *IL-10* haplotype and plasma IFN-γ expression

Haplotype		n	Median plasma IFN-γ (IQR)	*p*-value
AAT	No	23	11.83 (6.89 - 23.40)	0.94
	Yes	14	12.06 (8.48 - 17.68)	
CGA	No	16	11.55 (8.35 - 16.05)	0.63
	Yes	21	16.26 (7.17 - 23.40)	
CGT	No	23	11.42 (8.05 - 16.84)	0.58
	Yes	14	16.73 (4.87 - 26.04)	

Table 4.3.13 Association between *IL-10* haplotype and plasma TNF-α expression

Haplotype		n	Median plasma TNF-α (IQR)	*p*-value
AAT	No	25	15.65 (13.42 - 22.92)	0.46
	Yes	14	14.46 (13.72 - 17.08)	
CGA	No	16	14.46 (12.89 - 19.37)	0.37
	Yes	23	15.65 (13.42 - 24.18)	
CGT	No	25	14.86 (13.79 - 21.89)	0.93
	Yes	14	14.82 (11.89 - 22.92)	

4.4 DISCUSSION

Previous studies on the potent, immunoregulatory cytokine interleukin-10 have focused mainly on the polymorphisms and their role in HIV-1 susceptibility and rate of disease progression. The underlying mechanisms which may be associated with these polymorphisms have not been studied in an African setting of chronic HIV-1 infection.

With the onset of HIV-1 infection and rapid viral replication, chronic stimulation by HIV results in generalised activation of T cells. The increase in T cell proliferation and expansion dramatically depletes the $CD4^+$ T cell population, eventually leading to immunologic exhaustion and death. IL-10 has been shown to inhibit pro-inflammatory cytokines, and suppresses T cell function.

Mechanistic studies in the LCMV mouse model show that the blockade of IL-10 resulted in enhanced T cell responses (Brooks et al., 2006, Ejrnaes et al., 2006). In a more recent study, the blockade of IL-10 in PBMCs from HIV-infected individuals *in vitro*, resulted in robust proliferative and effector $CD4^+$ T cell function (Brockman et al., 2009).

This part of the study aimed to investigate the mechanistic role of IL-10 in chronic HIV infection. *IL-10* genotypes were characterised in individuals with chronic HIV-1C infection. In a subset of these individuals, we measured select markers of activation on $CD4^+$ T cells, $CD8^+$ T cells, and B cells. Interestingly, we found that the -592AA genotype (associated with low IL-10 production) and the -1082AA genotype (associated with low IL-10 production), were

significantly associated with increased expression of HLA-DR in $CD4^+$ T cells and $CD8^+$ T cells respectively. This has not been previously demonstrated based on *IL-10* promoter polymorphisms in an HIV setting. These results suggest that with decreased levels of IL-10, there is more expression of HLA-DR on the surface. As HLA-DR is a marker for immune activation, this suggests that with lower levels of IL-10, this allows for increased immune activation.

However, the -592AA genotype (associated with low IL-10 production) is also associated with higher levels of CD95 and PD-1 in $CD4^+$ T cells. CD-95 is a death receptor found on the surface of cells (Wajant, 2002). As PD-1 is a marker of immune exhaustion on $CD8^+$ T cells, further studies are required to determine the role of PD-1 expression on $CD4^+$ T cells.

The -592CC genotype (associated with high IL-10 production) and the -1082GG genotype (associated with high IL-10 production) were associated with increased expression of IgG on the surface of B cells. IgG has been shown to be involved in the secondary immune response. These results suggest that high IL-10 levels will result in an increased secondary immune response. These data may suggest that IL-10 shifts the immune response towards a Th2 phenotype associated with a robust humoral response. Therefore, IL-10 may reduce immune activation in chronic HIV-1 infection but bias the immune response toward Th2 phenotype. Th2 biased responses are thought to be detrimental in HIV-1 infection (Breytenbach et al., 2001, Clerici and Shearer, 1993, Rizzardi et al., 1998). In summary, our observations here suggest a complex and perhaps paradoxical role for IL-10 with reduced immune activation being beneficial while a Th2 bias may be detrimental in the control of this chronic viral infection.

Taken together these results suggest that higher levels of IL-10 down-regulates the expression of HLA molecules on the surface of cells and dampens immune activation, which is known to be one of the main drivers of disease progression in HIV-1 infection. However, the role of IL-10 is complex and lower levels of IL-10 may result in increased cell death (loss in $CD4^+$ T cells), while higher levels of IL-10 may be beneficial to the secondary immune response.

As IL-10 has been implicated in the inhibition of T cell proliferation, which leads to the dampening of T cell function, IL-10 receptor blockade assays were performed to see if this resulted in $CD4^+$ T cell proliferation. As these assays were performed on frozen PBMCs, the viability of the cells varied, and affected the outcome of these assays. Although we did not see any significant association with proliferation after IL-10 receptor blockade, there did however, seem to be a trend towards an increase in $CD4^+$ T cell proliferation after blockade. We may have not had adequate sample size to reach significance here. This may suggest that IL-10 has the ability to suppress proliferation.

Previous studies done on HIV controllers show that these individuals maintain high levels of IL-2 secreting cells, which proliferate when exposed to HIV peptides (Emu et al., 2005, Rosenberg et al., 1997). Therefore, we wanted to determine if IL-10 receptor blockade had any effect on cytokine production. We measured the levels of cytokines after IL-10 receptor blockade and found that consistently, IL-2 production was increased after IL-10 receptor blockade. This suggests that IL-10 inhibits the production of cytokines which play a significant role in the immune response. And that the blockade of IL-10 may result in enhanced production of these cytokines. *IL-10* haplotype did not associate with proliferation after IL-10 blockade. IL-2 was the

only cytokine that associated with *IL-10* haplotypes after IL-10 blockade. Individuals with the AAT haplotype, and individuals without the CGT haplotype had increased levels of IL-2 production following IL-10 blockade.

Plasma cytokine measurements did not significantly associate with extreme *IL-10* genotypes or haplotypes. We did not expect to see an effect on cytokine production, as samples included in this analysis were taken from individuals at varying timepoints. Also, co-infections may have affected the production of pro- and anti-inflammatory cytokines, masking the effect of these IL-10 variants.

Overall, IL-10 and its genetic variants seem to have a complex role in HIV-1 pathogenesis. Results suggest that lower IL-10 levels may favour cell death, while on the other hand IL-10 blockade may result in increased cell proliferation and IL-2 production. Better understanding of these underlying mechanisms may help to better understand immune activation and dysfunction in HIV-1 infection.

Chapter 5

Overall Discussion and Conclusion

It has been 30 years since the human immunodeficiency virus (HIV) was first described, and the resulting epidemic has since risen to pandemic proportions. The global epidemic of HIV-1 infection has resulted in high morbidity and mortality. The global distribution of HIV-1 infection is disproportionate, with almost two-thirds of the world's infected population found in the sub-Saharan African region, and many factors may contribute to this uneven distribution. Human genetic factors have been implicated in this disproportionate distribution, these are known as host restriction factors.

The cytokine interleukin-10 (IL-10) has been demonstrated to play a role in HIV-1 susceptibility and pathogenesis (Erikstrup et al., 2007, Naicker et al., 2009, Shin et al., 2000). Genetic studies suggest that polymorphisms associated with low IL-10 production were associated with an increased risk in HIV infection and with an acceleration to AIDS. However, the underlying mechanism of the role of IL-10 is not well understood. Recent studies on the LCMV mouse model show that the blockade of IL-10 results in enhanced T cell responses (Brooks et al., 2006, Ejrnaes et al., 2006). More recent studies show that IL-10 blockade in the PBMCs of HIV-infected individuals *in vitro* showed an increase in proliferative and effector $CD4^+$ T cell function (Brockman et al., 2009).

To explain the difference in the effects of these mutations we hypothesised the following models from our preliminary results (Naicker et al., 2009). With regards to HIV susceptibility, the high expression of IL-10 will lead to general inactivation of T lymphocytes, rendering them less susceptible to HIV-1 infection (see Figure 5.1). On the other hand, low IL-10 production is likely to result in increased T cell activation, thereby making the HIV target cells more

susceptible to HIV infection. Individuals who are genetically predisposed to low IL-10 production will therefore be more likely to have activated T cells particularly when they become infected with other infectious pathogens such as those which lead to sexually transmitted infections.

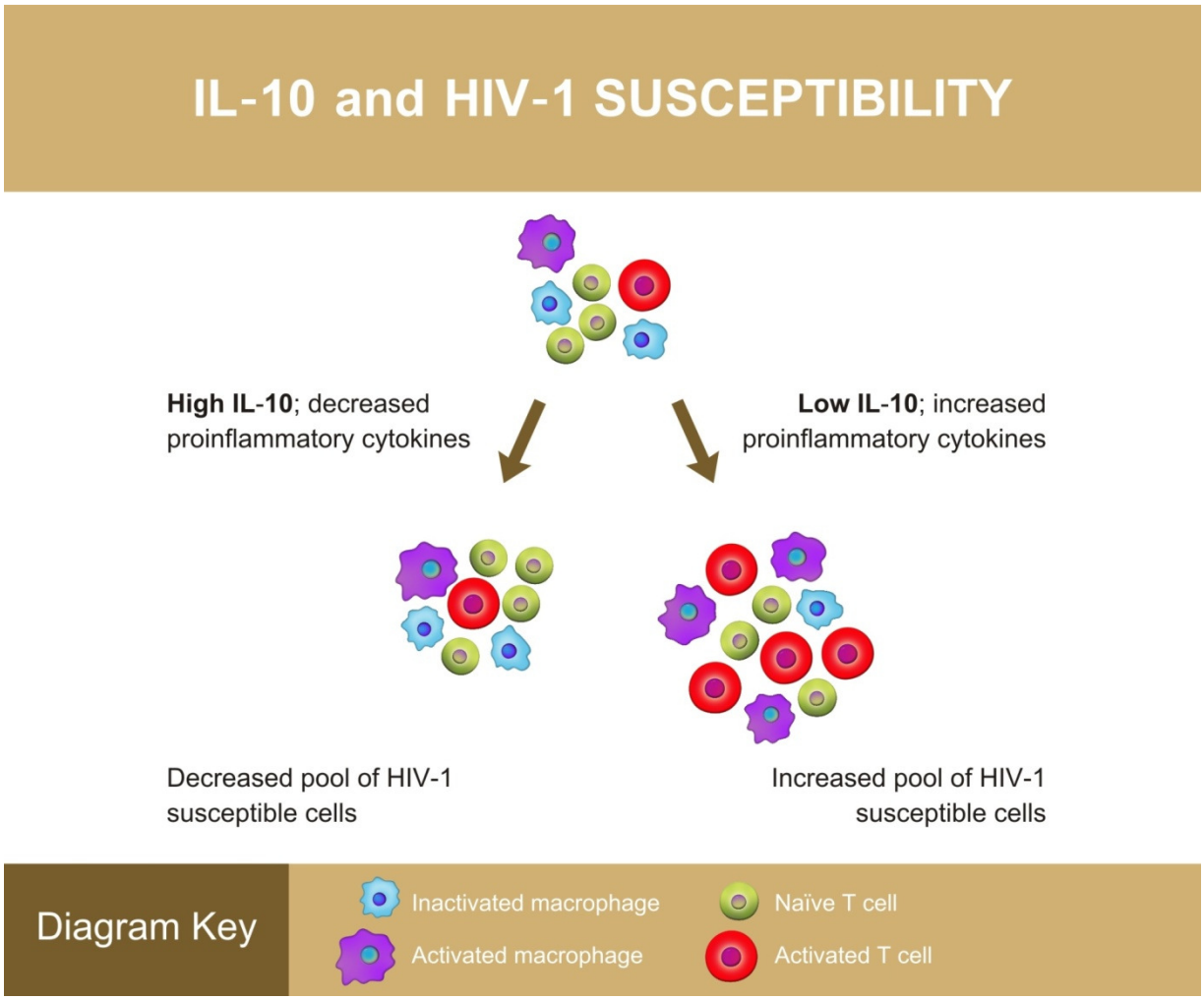

Figure 5.5 The role of IL-10 in HIV susceptibility

Once infected the role of IL-10 is complex and seems to be dependent on the phase of infection (see Figure 5.2). We hypothesise that during acute infection higher levels of IL-10 expression will result in decreased mobilisation, activation and proliferation of immune effector cells, this leads to a higher rate of viral replication. However, during the chronic phase of infection, HIV replication is more productive in macrophages as there is dramatic depletion of $CD4^+$ T cells. Therefore, in the chronic phase of infection higher IL-10 levels result in decreased immune activation and direct inhibition of viral replication within macrophages.

To test these hypotheses and to determine the effects and mechanisms of interleukin-10 promoter polymorphisms in HIV-1 susceptibility and pathogenesis, we aimed to characterise previously described *IL-10* promoter SNPs in HIV-negative individuals at high risk for HIV infection, and HIV-positive individuals in the acute and chronic phase of HIV-1 infection.

Three *IL-10* promoter polymorphisms, i.e. -592, -1082 and -3575, were characterised in a total of 685 individuals. These polymorphisms were found in different ethnic groups in previous genetic studies, and we found the presence of these polymorphisms in our South African cohort. The distal -3575 SNP was characterised in individuals from the CAPRISA Acute Infection cohort, where individuals at high risk for HIV-1C infection were followed up over time, and if they seroconverted they entered into phase two of the study where routine viral load and CD4 measurements were done at scheduled visits. There was no significance between the -3575 genotype and time to infection. However, we observed a trend that individuals with the -3575AA genotype (previously shown to be associated with low IL-10 production), were more likely to become HIV-infected, although this did not reach significance.

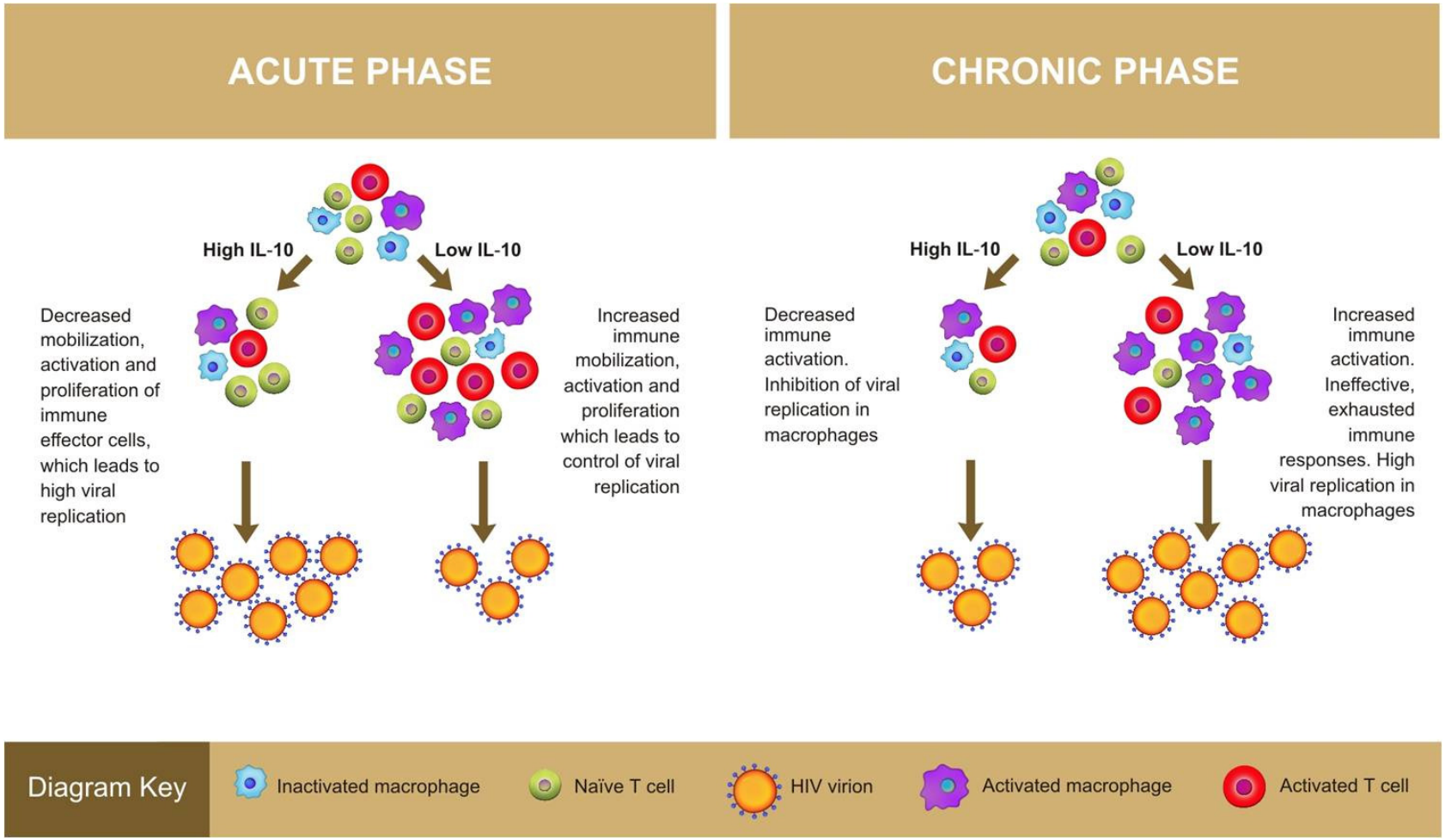

Figure 5.6 The role of IL-10 in acute and chronic HIV infection

We determined the association between the three *IL-10* genetic variants and biomarkers of HIV infection (Naicker et al., 2012). Here we investigated the association between *IL-10* variants and viral load, $CD4^+$ T cell count, and the rate of CD4 decline. We found no association between any *IL-10* genotype and either viral load or $CD4^+$ T cell count. Different haplotypes associated with viral load and $CD4^+$ T cell count in the CAPRISA Acute Infection cohort, and further analysis is required to determine how these haplotypes exert their effect. However, we found that *IL-10* genetic variants associated with low IL-10 production showed an attenuated loss of $CD4^+$ T cells during the first 24 months of follow-up, this did not reach significance for the -1082 genotype, but was significant for the -592 genotype. Interestingly the CGT haplotype was associated with a protection against HIV acquisition, as all the individuals with this haplotype were HIV-negative. This is possibly due to the association of high IL-10 production, with the alleles that make up this genotype.

We hypothesised that during the early stages of infection, high-IL-10-producing genotypes (and by extension higher IL-10 levels) can dampen the antiviral adaptive and innate effector mechanisms, resulting in poor control of viral replication (Alter et al., 2010, Brooks et al., 2006, Herbein and Varin, 2010, Martinic and von Herrath, 2008). However, the beneficial effects of IL-10 are more pronounced during the later stages of infection by its anti-inflammatory effects and the direct inhibition of HIV-1 replication within macrophages (Ancuta et al., 2001, Bento et al., 2009, Wang and Rice, 2006).

Previous studies have shown that *IL-10* promoter polymorphisms affect the expression of IL-10, however this had not been investigated in an HIV setting (Edwards-Smith et al., 1999, Eskdale et

al., 1998, Hutchinson et al., 1998, Turner et al., 1997). We investigated the effect of *IL-10* SNPs on IL-10 expression in an African setting of HIV infection. Our analysis showed that the -1082GG genotype, previously shown to associate with high IL-10 production, associated with high IL-10 levels in an HIV setting. We did not see any significant associations with the -592 genotype, but we saw a trend towards the -592AA genotype having lower IL-10 expression. The CAT haplotype showed an association with IL-10 expression, where individuals without the haplotype showed higher expression of IL-10, i.e. individuals carrying the A at position -1082 had lower levels of IL-10 expression.

The levels of IL-10 did not correlate with any of the biomarkers of HIV infection, such as viral load, $CD4^+$ T cell count, and the breadth and magnitude of immune responses. However, the time of infection was not known in individuals included in this part of the analysis, therefore we may be looking at a cross-section of individuals at different timepoints of infection, and therefore are not able to see a distinct association between IL-10 levels and these biomarkers of HIV infection.

The cytokine network is a precise balance of signals and feedback mechanisms. We investigated the effect of IL-10 levels on other pro-inflammatory cytokines such as IFN-γ, IL-2, IL-6 and TNF-α. Overall, we found that there was a significant positive correlation between IL-10 expression and these cytokines, showing that during the chronic phase of HIV infection, there seems to be a generalised upregulation of both pro- and anti-inflammatory cytokines. IL-10 also seemed to dominate the proportion of cytokine production during chronic infection suggesting its anti-inflammatory properties as pro-inflammatory cytokine production increases.

IL-10 is a pleiotropic cytokine involved in the immune response. These SNPs had not been investigated with regards to its role in the breadth and magnitude of immune response during the chronic phase of infection. Here we found that the low-IL-10-producing -592AA genotype is significantly associated with a larger number of HIV-specific peptides targeted by cytotoxic T lymphocytes (CTLs). This genotype also associated with an attenuated loss of $CD4^+$ T cells. Individuals with the CAA haplotype had a significantly greater magnitude of immune responses, as well as a significantly larger breadth of immune responses, as compared to individuals without the CAA haplotype, in the Sinikithemba Chronic Infection cohort. Expression analysis showed that individuals with the CAA haplotype tended to have a lower median IL-10 expression, however this was not significant. This suggests that lower levels of IL-10 may favour a greater magnitude of immune responses, and a larger number of peptides targeted by CTL.

HIV infection is characterised by a phase of rapid viral replication. Generalised T cell activation results in rapid T cell activation and proliferation, leading to exhaustion. As IL-10 has been shown to inhibit proliferation and higher levels of IL-10 have been demonstrated in individuals with severely compromised T helper cell function (Clerici et al., 1994, Ostrowski et al., 2001), we investigated the role of IL-10 receptor blockade on activation, proliferation and cytokine production. Markers of activation were measured in $CD4^+$ T cells, $CD8^+$ T cells and B cells. Interestingly, we found that in an HIV setting, the HLA-DR expression was significantly increased in individuals displaying the low-IL-10-producing -592AA and -1082AA genotypes. The higher levels of HLA-DR expression suggests that there is increased immune activation in individuals with low-IL-10-producing variants. This is particularly interesting as it suggests that

during chronic infection, higher levels of IL-10 may be beneficial, as it has the potential to reduce immune activation.

We found that the markers of cell death and immune exhaustion, i.e. CD95 and PD-1 were upregulated in individuals with the low-IL-10-producing -592AA genotype. This may suggest that lower levels of IL-10 may during the chronic phase of HIV-1 infection, may result in negative regulation of the immune response and increased immune exhaustion. This further emphasises the potential for IL-10 to be beneficial during the chronic stage of infection as it may reduce immune exhaustion. In B cells, the IgG marker, which is associated with the secondary immune response, was increased in the -592CC genotype (associated with high IL-10 production). This suggests that the production of higher levels of IL-10 during the chronic phase of HIV-1 infection may result in an enhanced secondary humoral immune response. Although we found higher expression of IgG in B cells associated with high IL-10 producer genotypes, this area of research will require further investigation as the subclasses of this IgG was not determined and neither were titers of secreted IgG measured. However, the data may be indicative to Th2 bias in the immune response in high IL-10 producers. The impact of *IL-10* genotypes and especially of IL-10 on B cells and the humoral immune response warrant further investigation.

IL-10 receptor blockade showed a trend towards higher $CD4^+$ T cell proliferation, however this was not significant. Factors which may have influenced this outcome are the viability of the cells and the small sample size. However, we did find a significant upregulation of IL-2 expression

following IL-10 receptor blockade. This suggests that with lower levels of IL-10, the high levels of proliferating IL-2 secreting cells may result in HIV control.

There are, however, some limitations to this study. As the time since HIV-1 infection is not known for study participants in the Sinikithemba cohort, this may have introduced a survivor bias in analysis of *IL-10* polymorphisms since these have been shown to affect survival. Also, this may suggest that we may be analysing individuals at different phases of infection altogether, although we aimed to account for this by stratifying our data according to viral loads and $CD4^+$ T cell counts. This study also focused on three *IL-10* promoter polymorphisms, only a subset of *IL-10* SNPs shown to affect IL-10 production. IL-10 and other cytokines can be induced by various other pathogens and co-infection data was not available for all individuals in this study.

With regards to our previous models from our preliminary data, the hypothesis still stands that individuals with genotypes associated with higher IL-10 levels are less susceptible to HIV infection compared to individuals with genotypes associated with lower IL-10 levels, although in this study we focused on pathogenesis following infection. During the acute phase of infection we found similar results based on the Acute Phase hypothesis, i.e. individuals with genotypes associated with higher IL-10 production will have a more dampened antiviral, T-cell adaptive and innate effector mechanisms, which in turn results in poor control of viral replication. Based on the results from this study, we can now update the hypothesis for the role of IL-10 in chronic infection (see Figure 5.3). Based on results from our study, we hypothesise that during chronic infection individuals with higher levels of IL-10 show an increase in the secondary immune response (IgG), which may lead to better control of viral replication. On the other hand,

individuals with lower levels of IL-10 have elevated levels of immune activation (HLA-DR expression) defective immune signaling in $CD4^+$ T cells leading to poor control of HIV replication.

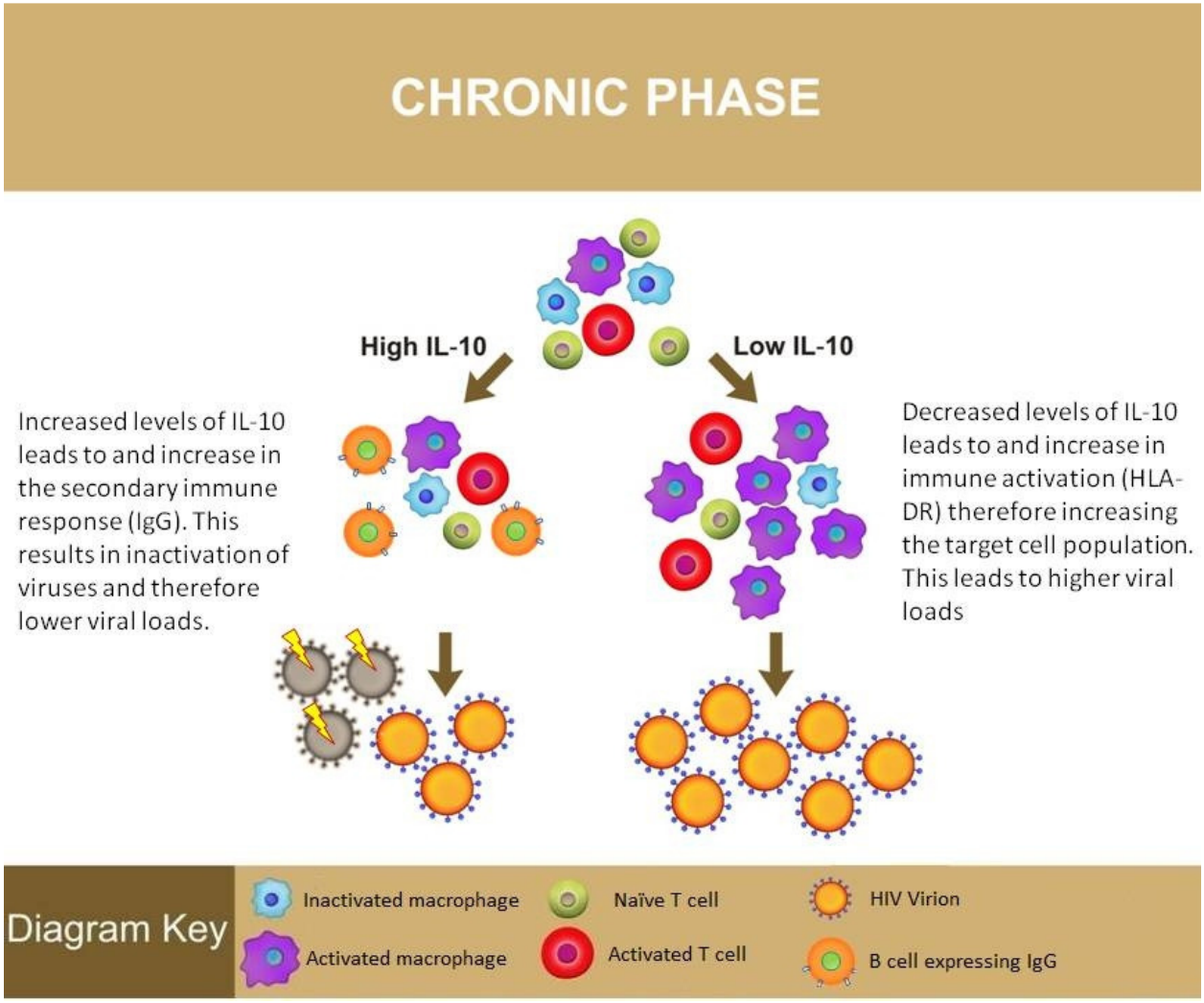

Figure 5.3 The role of IL-10 in chronic HIV-1 infection

Overall, IL-10 seems to play a complex role against HIV infection and pathogenesis. IL-10 seems to protect against infection, but its role during HIV-infection seems to be phase-dependant. *IL-10* genetic variants may be in linkage disequilibrium with other genes with a modulatory role, and may have underlying undetermined epigenetic or environment modulatory factors that affect HIV-susceptibility and pathogenesis.

Understanding the underlying factors and mechanisms by which IL-10 plays its role is essential in the development of an effective vaccine. A recent study investigating HIV-specific antibody-dependant cellular cytotoxicity (ADCC), showed that IL-10 enhanced the ability of natural killer cells to respond to HIV-specific ADCC antibodies (Wren et al., 2012). The results from our research add to the growing body and increasing interest into the role of IL-10 in HIV susceptibility and pathogenesis. This data suggests that overall, there is an upregulation of both pro- and anti-inflammatory cytokines, however IL-10 seems to dominate the proportion of production. The anti-inflammatory effect of IL-10 seems more evident during the chronic stages of infection in a setting of increased immune activation and immune exhaustion.

In order to fully understand the effects and mechanisms of *IL-10* promoter polymorphisms on HIV susceptibility and pathogenesis, expanded SNP analysis and additional mechanistic and functional studies should be performed on larger sample sizes. The aim of expanding the SNP analysis would be to include other *IL-10* promoter polymorphisms that are found further upstream from the transcription start site, in the distal region. This expanded SNP analysis will provide a better understanding of the extended haplotypes and their role in IL-10 production. Future analysis should ideally include larger sample sizes, including individuals at different

stages of HIV- infection, such as the acute stage of infection. This will help us to understand the role of IL-10 during different stages of HIV infection. Mechanistic and functional studies should include larger sample sizes for the activation and proliferation assays. This assay could be further strengthened by investigating the role of IL-10 by both receptor blockade, as well as supplementing IL-10 levels.

Understanding the role of this potent, anti-inflammatory cytokine and its polymorphisms may lead to a deeper understanding of the complex role of this gene in the immune response, which will help us understand how to target the IL-10 pathway for effective therapeutic or vaccine strategies.

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Appendix 1

Genotype and Haplotype Data for All Study Participants

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
HAI031	CC	AG	TA	CAT	CGA
HAI703	CA	AG	TT	CGT	AAT
HAI919	CC	AG	TA	CAT	CGA
HAI323	CC	GG	TA	CGT	CGA
HAI876	CC	AG	TA	CAT	CGA
HAI016	CC	AG	TA	CAT	CGA
HAI050	CA	AG	TA	CGA	AAT
HAI110	CA	AG	TA	CGA	AAT
HAI184	CC	AG	AA	CAA	CGA
HAI207	CA	AA	TT	CAT	AAT
HAI174	AA	AA	TT	AAT	
HAI341	CC	AG	TA	CAT	CGA
HAI358	CC	AG	TA	CAT	CGA
HAI483	CA	AG	TT	CGT	AAT
HAI802	AA	AA	TT	AAT	
HAI945	CA	AA	TT	CAT	AAT
HAI973	CC	AA	TT	CAT	
HAI017	CA	AG	TT	CGT	AAT
HAI1037	CC	GG	TA	CGT	CGA
HAI268	CC	AG	TA	CAT	CGA
HAI369	CC	GG	AA	CGA	
HAI458	CA	AG	TT	CGT	AAT
HAI513	CC	AA	TT	CAT	

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK001	CA	AA	TT	CAT	AAT
SK002	CC	AG	TA	CAT	CGA
SK003	CC	AG	TA	CAT	CGA
SK004	CA	AG	TA	CGA	AAT
SK005	CA	AA	TT	CAT	AAT
SK006	CC	AG	TA	CAT	CGA
SK007	CC	AA	TT	CAT	
SK008	CC	GG	AA	CGA	
SK009	AA	AA	TT	AAT	
SK010	CC	AA	TT	CAT	
SK011	AA	AA	TT	AAT	
SK012	AA	AA	TT	AAT	
SK013	CA	AG	TA	CGA	AAT
SK014	CA	AA	TT	CAT	AAT
SK015	CC	AA	TT	CAT	
SK016	CC	AG	TT	CAT	CGT
SK017	CA	AG	TA	CGA	AAT
SK018	CA	AA	TT	CAT	AAT
SK019	AA	AA	TT	AAT	
SK020	CA	AG	TA	CGA	AAT
SK021	AA	AA	TT	AAT	
SK022	AA	AA	TT	AAT	
SK023	CC	AA	TT	CAT	
SK024	CC	AG	TA	CAT	CGA
SK025	CC	GG	TA	CGT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK026	CC	-	AA		
SK027	CC	GG	TA	CGT	CGA
SK028	CC	AG	TA	CAT	CGA
SK029	CC	AA	TT	CAT	
SK030	CC	GG	TA	CGT	CGA
SK031	CC	AG	TT	CAT	CGT
SK032	CC	AA	TT	CAT	
SK033	CA	AA	TT	CAT	AAT
SK034	AA	AA	TT	AAT	
SK035	CC	GG	TA	CGT	CGA
SK036	CA	AA	TT	CAT	AAT
SK037	CC	GG	TA	CGT	CGA
SK038	CC	AG	TA	CAT	CGA
SK039	CA	AA	TT	CAT	AAT
SK040	CA	AG	TT	CGT	AAT
SK041	CC	AG	TA	CAT	CGA
SK042	CC	AG	TA	CAT	CGA
SK043	CA	AA	TT	CAT	AAT
SK044	CC	GG	TA	CGT	CGA
SK045	CA	AA	TT	CAT	AAT
SK046	CA	AA	TT	CAT	AAT
SK047	CC	GG	TA	CGT	CGA
SK048	AA	AA	TT	AAT	
SK049	CA	AA	TT	CAT	AAT
SK050	CC	AG	TT	CAT	CGT
SK051	CC	AG	TT	CAT	CGT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK052	CA	AA	TT	CAT	AAT
SK053	CA	AG	TA	CGA	AAT
SK054	CC	AA	TT	CAT	
SK055	CC	AG	TA	CAT	CGA
SK056	CA	AA	TT	CAT	AAT
SK057	CC	-	AA		
SK058	CC	AG	TA	CAT	CGA
SK059	CA	AG	TA	CGA	AAT
SK060	CA	AG	TA	CGA	AAT
SK061	CC	GG	TA	CGT	CGA
SK062	CA	AG	TA	CGA	AAT
SK063	CA	AA	TT	CAT	AAT
SK064	CC	AG	TA	CAT	CGA
SK065	AA	AA	TT	AAT	
SK066	AA	AA	TT	AAT	
SK067	CC	AG	TT	CAT	CGT
SK068	CC	AG	TA	CAT	CGA
SK069	CA	AG	TA	CGA	AAT
SK070	CA	AG	TT	CGT	AAT
SK071	CA	AG	TA	CGA	AAT
SK072	AA	AA	TT	AAT	
SK073	CA	AG	TA	CGA	AAT
SK074	CC	GG	TA	CGT	CGA
SK075	CA	AA	TT	CAT	AAT
SK076	CA	AG	TT	CGT	AAT
SK077	CA	AA	TT	CAT	AAT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK078	CA	AA	TT	CAT	AAT
SK079	CA	AG	TT	CGT	AAT
SK080	CC	AA	TT	CAT	
SK081	CC	AG	TA	CAT	CGA
SK082	CA	AA	TT	CAT	AAT
SK083	AA	AA	TT	AAT	
SK084	AA	AA	TT	AAT	
SK085	CC	AG	TA	CAT	CGA
SK086	AA	AA	TT	AAT	
SK087	CA	AG	TA	CGA	AAT
SK088	CC	GG	AA	CGA	
SK089	CC	AG	TA	CAT	CGA
SK090	CA	AA	TA	CAA	AAT
SK091	CA	AA	TT	CAT	AAT
SK092	CC	AA	TT	CAT	
SK093	CA	AG	TA	CGA	AAT
SK094	CC	AG	TA	CAT	CGA
SK095	CA	AG	TA	CGA	AAT
SK096	CA	AA	TT	CAT	AAT
SK097	CA	AA	TT	CAT	AAT
SK098	CA	AG	TA	CGA	AAT
SK099	CC	AG	TA	CAT	CGA
SK100	CA	AA	TT	CAT	AAT
SK101	CC	AG	TA	CAT	CGA
SK102	CA	AA	TT	CAT	AAT
SK103	AA	AA	TT	AAT	

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK104	CC	GG	AA	CGA	
SK105	CC	AG	TA	CAT	CGA
SK106	CA	AA	TT	CAT	AAT
SK107	AA	AA	TT	AAT	
SK108	CA	AG	TA	CGA	AAT
SK109	CA	AA	TA	CAA	AAT
SK110	CA	AG	TA	CGA	AAT
SK111	CC	AA	TT	CAT	
SK112	CC	AA	TT	CAT	
SK113	CA	AA	TT	CAT	AAT
SK114	CC	AG	TT	CAT	CGT
SK115	AA	AA	TT	AAT	
SK116	CC	AA	TT	CAT	
SK117	AA	AA	TT	AAT	
SK118	CA	AA	TT	CAT	AAT
SK119	CC	AA	TT	CAT	
SK120	CA	AG	TT	CGT	AAT
SK121	CA	AG	TT	CGT	AAT
SK122	CC	AG	TA	CAT	CGA
SK123	CA	AG	TT	CGT	AAT
SK124	CC	AG	TA	CAT	CGA
SK125	AA	AA	TT	AAT	
SK126	CA	AG	TA	CGA	AAT
SK127	CA	AG	TA	CGA	AAT
SK128	CA	AA	TT	CAT	AAT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK129	AA	AA	TT	AAT	
SK130	CA	AG	TA	CGA	AAT
SK131	CC	AG	TT	CAT	CGT
SK132	AA	AA	TT	AAT	
SK133	CA	AG	TA	CGA	AAT
SK134	CA	AG	TT	CGT	AAT
SK135	CA	AA	TT	CAT	AAT
SK136	CA	AG	TT	CGT	AAT
SK137	AA	AA	TT	AAT	
SK138	AA	AA	TT	AAT	
SK139	CA	AA	TT	CAT	AAT
SK140	CC	AG	TT	CAT	CGT
SK141	CA	AA	TT	CAT	AAT
SK142	CC	GG	TA	CGT	CGA
SK143	CC	AA	TT	CAT	
SK144	CA	AA	TT	CAT	AAT
SK145	AA	AA	TT	AAT	
SK146	CA	AA	TT	CAT	AAT
SK147	CC	GG	AA	CGA	
SK148	CC	AA	TT	CAT	
SK149	CA	AA	TT	CAT	AAT
SK150	AA	AA	TT	AAT	
SK151	CA	AG	TA	CGA	AAT
SK152	AA	AA	TT	AAT	
SK153	CA	AG	TA	CGA	AAT
SK154	CA	AG	TA	CGA	AAT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK155	CC	AA	TA	CAT	CAA
SK156	CA	AA	TT	CAT	AAT
SK157	CA	AG	TA	CGA	AAT
SK158	CC	GG	TA	CGT	CGA
SK159	CA	AG	TT	CGT	AAT
SK160	CA	AA	TT	CAT	AAT
SK161	CC	AG	TA	CAT	CGA
SK162	CC	AG	TT	CAT	CGT
SK163	CC	AA	TT	CAT	
SK164	CA	AA	TT	CAT	AAT
SK165	CC	GG	AA	CGA	
SK166	CC	AA	TT	CAT	
SK167	CC	AG	TT	CAT	CGT
SK168	CA	AG	TA	CGA	AAT
SK169	CA	AG	TA	CGA	AAT
SK170	CA	AA	TT	CAT	AAT
SK171	CC	AA	TT	CAT	
SK172	CC	AG	TT	CAT	CGT
SK173	AA	AA	TT	AAT	
SK174	CC	GG	AA	CGA	
SK175	CC	AA	TT	CAT	
SK176	CC	AG	TA	CAT	CGA
SK177	CA	AG	TT	CGT	AAT
SK178	CA	AG	TA	CGA	AAT
SK179	CA	AA	TT	CAT	AAT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK180	CA	AG	TA	CGA	AAT
SK181	CC	AG	TT	CAT	CGT
SK182	CA	AA	TT	CAT	AAT
SK183	CA	AG	TA	CGA	AAT
SK184	AA	AA	TT	AAT	
SK185	CA	AG	TA	CGA	AAT
SK186	CC	GG	TA	CGT	CGA
SK187	CA	AA	TT	CAT	AAT
SK188	CC	GG	TA	CGT	CGA
SK189	CA	AG	TA	CGA	AAT
SK190	CC	AG	TA	CAT	CGA
SK191	CA	AG	TT	CGT	AAT
SK192	CA	AA	TT	CAT	AAT
SK193	CC	AA	TT	CAT	
SK194	CA	AA	TT	CAT	AAT
SK195	CA	AG	TA	CGA	AAT
SK196	CC	AA	TT	CAT	
SK197	CA	AA	TT	CAT	AAT
SK198	CA	AG	TA	CGA	AAT
SK199	CC	AG	TA	CAT	CGA
SK200	CA	AA	TT	CAT	AAT
SK201	CC	GG	TA	CGT	CGA
SK202	CA	AA	TT	CAT	AAT
SK203	CA	AG	TA	CGA	AAT
SK204	CC	AG	TA	CAT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK205	CC	GG	TA	CGT	CGA
SK206	CC	AA	TT	CAT	
SK207	CC	GG	TA	CGT	CGA
SK208	CC	AG	TA	CAT	CGA
SK209	CA	AG	TA	CGA	AAT
SK210	CC	AA	TT	CAT	
SK211	CA	AA	TT	CAT	AAT
SK212	CA	AA	TT	CAT	AAT
SK213	CC	AA	TT	CAT	
SK214	AA	AA	TT	AAT	
SK215	CA	AA	TT	CAT	AAT
SK216	CA	AA	TT	CAT	AAT
SK217	CA	AA	TT	CAT	AAT
SK218	AA	AA	TT	AAT	
SK219	AA	AA	TT	AAT	
SK220	CC	AA	TT	CAT	
SK221	CC	GG	TA	CGT	CGA
SK222	CC	AG	TA	CAT	CGA
SK223	CA	AA	TT	CAT	AAT
SK224	CA	AG	TA	CGA	AAT
SK225	CC	AA	TT	CAT	
SK226	CC	AG	TA	CAT	CGA
SK227	CA	AG	TA	CGA	AAT
SK228	CC	AA	TT	CAT	
SK229	CC	AA	TT	CAT	
SK230	CC	AG	TA	CAT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK231	CA	AA	TA	CAA	AAT
SK232	CC	AG	TA	CAT	CGA
SK233	CA	AG	TA	CGA	AAT
SK234	CA	AG	TA	CGA	AAT
SK235	CC	GG	AA	CGA	
SK236	CC	AG	TA	CAT	CGA
SK237	CC	AA	TT	CAT	
SK238	CA	AG	TT	CGT	AAT
SK239	AA	AA	TT	AAT	
SK240	CC	AG	TA	CAT	CGA
SK241	CA	AG	TT	CGT	AAT
SK242	CC	AG	TA	CAT	CGA
SK243	CA	AA	TT	CAT	AAT
SK244	CC	AA	TT	CAT	
SK245	CC	AA	TT	CAT	
SK246	CA	AA	TT	CAT	AAT
SK247	CC	AG	TT	CAT	CGT
SK248	CC	GG	AA	CGA	
SK249	CA	AG	TA	CGA	AAT
SK250	CC	AG	TT	CAT	CGT
SK251	CC	GG	TT	CGT	
SK252	CC	AA	TT	CAT	
SK253	CC	AA	TT	CAT	
SK254	CA	AA	TT	CAT	AAT
SK255	CC	AA	TT	CAT	

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK256	CC	AA	TT	CAT	
SK257	CA	AG	TA	CGA	AAT
SK258	CC	AA	TT	CAT	
SK259	CA	AG	TA	CGA	AAT
SK260	CC	AG	TA	CAT	CGA
SK261	CA	AG	TA	CGA	AAT
SK262	CC	AA	TT	CAT	
SK263	CA	AG	TT	CGT	AAT
SK264	CA	AA	TT	CAT	AAT
SK265	CA	AA	TT	CAT	AAT
SK266	CA	AA	TT	CAT	AAT
SK267	CC	AA	TT	CAT	
SK268	CA	AA	TT	CAT	AAT
SK269	CC	GG	AA	CGA	
SK270	CA	AA	TT	CAT	AAT
SK271	CA	AG	TA	CGA	AAT
SK272	CC	AG	TT	CAT	CGT
SK273	CA	AG	TT	CGT	AAT
SK274	CA	AA	TT	CAT	AAT
SK275	CA	AA	TT	CAT	AAT
SK276	AA	AA	TT	AAT	
SK277	CA	AA	TT	CAT	AAT
SK278	AA	AA	TT	AAT	
SK279	CC	AG	TA	CAT	CGA
SK280	CA	AG	TT	CGT	AAT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK281	CC	GG	TA	CGT	CGA
SK282	CA	AG	TA	CGA	AAT
SK283	CC	AA	TA	CAT	CAA
SK284	CC	AG	TA	CAT	CGA
SK285	CC	AG	TA	CAT	CGA
SK286	CA	AG	TA	CGA	AAT
SK287	CC	AG	TA	CAT	CGA
SK288	CC	AG	TA	CAT	CGA
SK289	CA	AA	TT	CAT	AAT
SK290	CA	AG	TA	CGA	AAT
SK291	CA	AA	TT	CAT	AAT
SK292	CA	AA	TT	CAT	AAT
SK293	CC	AG	TT	CAT	CGT
SK294	CA	AA	TT	CAT	AAT
SK295	CA	AA	TT	CAT	AAT
SK296	AA	AA	TT	AAT	
SK297	CA	AA	TA	CAA	AAT
SK298	CA	AG	TA	CGA	AAT
SK299	CA	AG	TT	CGT	AAT
SK300	CC	GG	AA	CGA	
SK301	CA	AG	TT	CGT	AAT
SK302	CA	AG	TT	CGT	AAT
SK303	CA	AA	TA	CAA	AAT
SK304	CC	AA	TT	CAT	
SK305	CA	AG	TA	CGA	AAT
SK306	AA	AA	TT	AAT	

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK307	CA	AA	TA	CAA	AAT
SK308	CC	GG	AA	CGA	
SK309	CA	AA	TT	CAT	AAT
SK310	AA	AA	TT	AAT	
SK311	CA	AA	TT	CAT	AAT
SK312	CC	AA	TT	CAT	
SK313	CC	GG	TA	CGT	CGA
SK314	CA	AG	TA	CGA	AAT
SK315	CA	AA	TT	CAT	AAT
SK316	CA	AA	TT	CAT	AAT
SK317	AA	AA	TT	AAT	
SK318	CA	AA	TT	CAT	AAT
SK319	CC	AG	TA	CAT	CGA
SK320	CA	AA	TT	CAT	AAT
SK321	CA	AA	TT	CAT	AAT
SK322	AA	AA	TT	AAT	
SK323	CC	AA	TT	CAT	
SK324	CA	AG	TA	CGA	AAT
SK325	CC	AA	TT	CAT	
SK326	CA	AG	TA	CGA	AAT
SK327	CC	AG	TA	CAT	CGA
SK328	CC	AA	TT	CAT	
SK329	CA	AA	TT	CAT	AAT
SK330	CA	AA	TT	CAT	AAT
SK331	CC	GG	TA	CGT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK332	CA	AA	TT	CAT	AAT
SK333	CA	AA	TT	CAT	AAT
SK334	CA	AG	TA	CGA	AAT
SK335	CC	AA	TT	CAT	
SK336	CA	AG	TA	CGA	AAT
SK337	CA	AG	TA	CGA	AAT
SK338	CC	AG	TA	CAT	CGA
SK339	CC	AG	TT	CAT	CGT
SK340	AA	AA	TT	AAT	
SK341	CC	AG	TA	CAT	CGA
SK342	CA	AA	TT	CAT	AAT
SK343	CA	AA	TT	CAT	AAT
SK344	CA	AG	TA	CGA	AAT
SK345	CA	AA	TT	CAT	AAT
SK346	CA	AG	TA	CGA	AAT
SK347	CA	AG	TA	CGA	AAT
SK348	CA	AG	TA	CGA	AAT
SK349	CC	GG	TA	CGT	CGA
SK350	CC	GG	TA	CGT	CGA
SK351	CA	AA	TT	CAT	AAT
SK352	CA	AA	TT	CAT	AAT
SK353	CC	AG	TT	CAT	CGT
SK354	CA	AA	TT	CAT	AAT
SK355	CC	AG	TT	CAT	CGT
SK356	CC	AG	TA	CAT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK357	CA	AG	TA	CGA	AAT
SK358	CC	GG	AA	CGA	
SK359	CC	AA	TT	CAT	
SK360	AA	AA	TT	AAT	
SK361	CA	AA	TT	CAT	AAT
SK362	CC	AA	TT	CAT	
SK363	CA	AG	TA	CGA	AAT
SK364	CA	AA	TT	CAT	AAT
SK365	CC	AG	AA	CAA	CGA
SK366	CC	AG	TT	CAT	CGT
SK367	CC	GG	AA	CGA	
SK368	CA	AG	TA	CGA	AAT
SK369	CC	AA	TT	CAT	
SK370	CC	AA	TT	CAT	
SK371	CC	AG	TA	CAT	CGA
SK372	AA	AA	TT	AAT	
SK373	CA	AG	TA	CGA	AAT
SK374	CC	AA	TT	CAT	
SK375	CA	AG	TA	CGA	AAT
SK376	CC	GG	TA	CGT	CGA
SK377	CA	AG	TT	CGT	AAT
SK378	CC	GG	TA	CGT	CGA
SK379	AA	AA	TT	AAT	
SK380	CC	AA	TT	CAT	
SK381	CA	AA	TT	CAT	AAT
SK382	CA	AG	TA	CGA	AAT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK383	CC	GG	TA	CGT	CGA
SK384	CC	AA	TT	CAT	
SK385	CA	AG	TT	CGT	AAT
SK386	CA	AG	TT	CGT	AAT
SK387	CC	GG	TA	CGT	CGA
SK388	CA	AG	TT	CGT	AAT
SK389	AA	AA	TT	AAT	
SK390	AA	AA	TT	AAT	
SK391	CC	AG	TA	CAT	CGA
SK392	CC	AG	TT	CAT	CGT
SK393	CC	AG	TT	CAT	CGT
SK394	CA	AA	TT	CAT	AAT
SK395	CA	AA	TT	CAT	AAT
SK396	CA	AA	TA	CAA	AAT
SK397	CC	AG	TA	CAT	CGA
SK398	CC	AA	TT	CAT	
SK399	CA	AA	TT	CAT	AAT
SK400	CA	AG	TT	CGT	AAT
SK401	AA	AA	TT	AAT	
SK402	CC	AA	TA	CAT	CAA
SK403	CC	AA	TA	CAT	CAA
SK404	CA	AA	TT	CAT	AAT
SK405	CC	AG	TA	CAT	CGA
SK406	CC	GG	AA	CGA	
SK407	CC	AG	TA	CAT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK408	AA	AA	TT	AAT	
SK409	CA	AA	TT	CAT	AAT
SK410	CA	AA	TT	CAT	AAT
SK411	CA	AA	TT	CAT	AAT
SK412	CC	AA	TT	CAT	
SK413	CC	GG	TA	CGT	CGA
SK414	CA	AG	TA	CGA	AAT
SK415	CA	AG	TT	CGT	AAT
SK416	CA	AG	TA	CGA	AAT
SK417	CC	AA	TT	CAT	
SK418	CA	AA	TT	CAT	AAT
SK419	AA	AA	TT	AAT	
SK420	CC	GG	TT	CGT	
SK421	CC	GG	AA	CGA	
SK422	CC	AG	TT	CAT	CGT
SK423	CC	AA	TT	CAT	
SK424	CC	GG	TT	CGT	
SK425	AA	AA	TT	AAT	
SK426	CA	AA	TT	CAT	AAT
SK427	CC	AG	TA	CAT	CGA
SK428	CA	AA	TT	CAT	AAT
SK429	-	AA	TT		
SK430	CC	AG	TA	CAT	CGA
SK431	CA	AG	TA	CGA	AAT
SK432	CA	AA	TT	CAT	AAT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
SK433	CC	AG	TA	CAT	CGA
SK434	CC	AG	TT	CAT	CGT
SK435	CA	AA	TT	CAT	AAT
SK436	CA	AG	TA	CGA	AAT
SK437	CA	AG	TA	CGA	AAT
SK438	CA	AA	TT	CAT	AAT
SK439	CC	AA	TT	CAT	
SK440	CA	AG	TA	CGA	AAT
SK441	CC	AG	TA	CAT	CGA
SK442	CA	AA	TT	CAT	AAT
SK443	CA	AA	TT	CAT	AAT
SK444	CC	GG	AA	CGA	
SK445	CA	AG	TA	CGA	AAT
SK446	CC	AG	TA	CAT	CGA
SK447	CA	AG	TT	CGT	AAT
SK448	CC	AA	TT	CAT	
SK449	CC	AA	TT	CAT	
SK450	CA	AG	TA	CGA	AAT
SK451	CC	AG	TA	CAT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0001	CA	AG	TA	CGA	AAT
CAI0002	CC	AG	TA	CAT	CGA
CAI0004	CC	AG	TA	CAT	CGA
CAI0006	CC	AA	TT	CAT	
CAI0008	AA	AA	TT	AAT	
CAI0009	CA	AG	TA	CGA	AAT
CAI0010	CA	AG	TT	CGT	AAT
CAI0011	CA	AA	TA	CAA	AAT
CAI0012	CC	AG	TT	CAT	CGT
CAI0015	CA	AG	TT	CGT	AAT
CAI0016	CC	AA	TT	CAT	
CAI0017	AA	AA	TT	AAT	
CAI0020	CC	AG	TT	CAT	CGT
CAI0021	AA	AA	TT	AAT	
CAI0022	CA	AA	TT	CAT	AAT
CAI0023	CC	AG	TA	CAT	CGA
CAI0024	CA	AG	TT	CGT	AAT
CAI0025	CA	AA	TT	CAT	AAT
CAI0027	AA	AA	TT	AAT	
CAI0028	AA	AA	TT	AAT	
CAI0029	CC	GG	TA	CGT	CGA
CAI0030	CA	AA	TT	CAT	AAT
CAI0031	CA	GG	TT	CGT	AGT
CAI0032	CC	AA	TT	CAT	
CAI0033	AA	AA	TT	AAT	
CAI0034	CC	AG	TA	CAT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0035	CC	AA	TT	CAT	
CAI0036	CA	AG	TT	CGT	AAT
CAI0037	CA	AA	TT	CAT	AAT
CAI0038	AA	AA	TT	AAT	
CAI0039	CA	AG	TT	CGT	AAT
CAI0040	CA	AA	TT	CAT	AAT
CAI0041	CC	GG	AA	CGA	
CAI0042	CC	AG	TT	CAT	CGT
CAI0044	CC	AG	TT	CAT	CGT
CAI0045	CC	AA	TT	CAT	
CAI0047	CC	AG	TA	CAT	CGA
CAI0048	CC	AG	TT	CAT	CGT
CAI0051	CA	AA	TT	CAT	AAT
CAI0052	CA	AG	TA	CGA	AAT
CAI0053	AA	AA	TT	AAT	
CAI0054	CC	AG	TA	CAT	CGA
CAI0055	CA	AA	TT	CAT	AAT
CAI0056	CA	AG	TT	CGT	AAT
CAI0057	CC	AG	TA	CAT	CGA
CAI0058	CA	GG	TT	CGT	AGT
CAI0059	CC	AA	TT	CAT	
CAI0060	AA	AA	TT	AAT	
CAI0061	CA	AA	TT	CAT	AAT
CAI0063	CC	AG	TT	CAT	CGT
CAI0065	CC	AA	TT	CAT	
CAI0066	CC	GG	TA	CGT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0067	CA	AA	TT	CAT	AAT
CAI0068	CC	AG	TT	CAT	CGT
CAI0069	CC	GG	AA	CGA	
CAI0070	CA	AA	TT	CAT	AAT
CAI0072	AA	AA	TT	AAT	
CAI0074	CC	AA	TT	CAT	
CAI0076	CA	AA	TT	CAT	AAT
CAI0077	CA	AA	TT	CAT	AAT
CAI0078	CA	AG	TA	CGA	AAT
CAI0080	CC	GG	TA	CGT	CGA
CAI0081	CC	AA	TT	CAT	
CAI0082	CC	AG	TA	CAT	CGA
CAI0083	AA	AA	TT	AAT	
CAI0084	CC	GG	AA	CGA	
CAI0085	CA	AA	TT	CAT	AAT
CAI0086	CA	AA	TT	CAT	AAT
CAI0087	CC	AG	TA	CAT	CGA
CAI0088	AA	AA	TT	AAT	
CAI0089	CA	AG	TA	CGA	AAT
CAI0091	CC	AA	TT	CAT	
CAI0092	CA	AA	TA	CAA	AAT
CAI0093	CA	AA	TT	CAT	AAT
CAI0094	CA	AA	TT	CAT	AAT
CAI0095	CA	AG	TA	CGA	AAT
CAI0096	CA	AA	TT	CAT	AAT
CAI0098	CA	AA	TT		
CAI0099	CA	AG	TA	CAT	AAT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0100	CA	AG	TT	CGA	AAT
CAI0101	CA	AA	TA	CGT	AAT
CAI0102	CC	GG	TT	CAA	AAT
CAI0103	CA	AA	TT	CGT	
CAI0106	CA	GG	TT	CAT	AAT
CAI0107	AA	AA	TT	CGT	AGT
CAI0108	CA	AA	TT	AAT	
CAI0109	CC	AA	TT	CAT	AAT
CAI0110	CC	AG	TA	CAT	
CAI0113	CA	GG	TA	CGA	AGT
CAI0114	CC	GG	TT	CGT	
CAI0115	CC	GG	AA	CGA	
CAI0116	CC	AG	TA	CAT	CGA
CAI0117	CC	GG	TA	CGT	CGA
CAI0120	CA	AG	TT	CGT	AAT
CAI0122	CA	AA	TT	CAT	AAT
CAI0123	CA	AA	TT	CAT	AAT
CAI0124	CC	AG	TA	CAT	CGA
CAI0125	CC	AG	TA	CAT	CGA
CAI0126	CA	AA	TT	CAT	AAT
CAI0128	CC	AA	TT	CAT	
CAI0129	CC	AG	AA	CAA	CGA
CAI0130	CC	AG	TT	CAT	CGT
CAI0131	CC	AG	TA	CAT	CGA
CAI0132	CA	AA	TT	CAT	AAT
CAI0133	CC	AA	TT	CAT	

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0134	CA	AA	TT	CAT	AAT
CAI0135	CA	AG	TA	CGA	AAT
CAI0136	CA	AA	TT	CAT	AAT
CAI0137	AA	AA	TT	AAT	
CAI0138	CA	AA	TT	CAT	AAT
CAI0139	CA	AA	TA	CAA	AAT
CAI0140	CA	AA	TT	CAT	AAT
CAI0141	CA	AG	TA	CGA	AAT
CAI0142	CA	AA	TT	CAT	AAT
CAI0143	CC	AG	TA	CAT	CGA
CAI0144	CC	AG	TT	CAT	CGT
CAI0145	CA	AG	TT	CGT	AAT
CAI0146	CC	AG	TA	CAT	CGA
CAI0147	CC	AA	TT	CAT	
CAI0148	CC	AG	TA	CAT	CGA
CAI0149	CC	AA	TT	CAT	
CAI0150	CA	AG	TT	CGT	AAT
CAI0151	CC	AA	TT	CAT	
CAI0152	CC	AG	TA	CAT	CGA
CAI0153	CC	AG	TA	CAT	CGA
CAI0154	CA	AG	TA	CGA	AAT
CAI0155	CA	AA	TT	CAT	AAT
CAI0156	CC	GG	TA	CGT	CGA
CAI0157	AA	AA	TT	AAT	
CAI0158	CC	AG	TA	CAT	CGA
CAI0159	AA	AA	TT	AAT	

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0161	CC	GG	TA	CGT	CGA
CAI0162	CC	AG	TT	CAT	CGT
CAI0163	CC	AA	TT	CAT	
CAI0164	CA	AG	TA	CGA	AAT
CAI0165	AA	GG	TT	AGT	
CAI0166	CA	AA	TT	CAT	AAT
CAI0167	CC	AA	TT	CAT	
CAI0168	AA	AA	TT	AAT	
CAI0169	CA	AG	TA	CGA	AAT
CAI0170	CA	AA	TT	CAT	AAT
CAI0171	CA	AA	TT	CAT	AAT
CAI0172	CC	AA	TT	CAT	
CAI0173	CA	GG	TA	CGA	AGT
CAI0174	AA	AA	TT	AAT	
CAI0175	CA	AA	TT	CAT	AAT
CAI0176	CC	GG	TT	CGT	
CAI0177	CA	AG	TA	CGA	AAT
CAI0178	CC	GG	TT	CGT	
CAI0179	AA	GG	TT	AGT	
CAI0181	CA	AG	TA	CGA	AAT
CAI0182	CC	AG	TA	CAT	CGA
CAI0183	CA	AA	TT	CAT	AAT
CAI0184	CC	AG	TA	CAT	CGA
CAI0185	CC	AA	TA	CAT	CAA
CAI0186	CA	AA	TT	CAT	AAT
CAI0187	CA	AA	TT	CAT	AAT
CAI0188	CA	AA	TT	CAT	AAT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0189	CC	AA	TT	CAT	
CAI0190	CA	AG	TA	CGA	AAT
CAI0191	CC	AA	TT	CAT	
CAI0192	CC	AG	TA	CAT	CGA
CAI0193	CC	AA	TT	CAT	
CAI0194	CA	AG	TA	CGA	AAT
CAI0195	CA	AG	TA	CGA	AAT
CAI0197	CA	AA	TT	CAT	AAT
CAI0198	CC	AA	TT	CAT	
CAI0199	CA	AA	TT	CAT	AAT
CAI0200	CC	AA	TT	CAT	
CAI0201	AA	AA	TT	AAT	
CAI0202	CA	AA	TT	CAT	AAT
CAI0203	CC	AG	TA	CAT	CGA
CAI0205	CA	AG	TA	CGA	AAT
CAI0206	CC	AA	TT	CAT	
CAI0207	CC	AG	TA	CAT	CGA
CAI0208	CA	AG	TA	CGA	AAT
CAI0209	CC	AG	TA	CAT	CGA
CAI0210	AA	AA	TT	AAT	
CAI0211	CA	AG	TA	CGA	AAT
CAI0212	CC	AG	TA	CAT	CGA
CAI0213	CC	GG	TT	CGT	
CAI0214	CC	AG	TA	CAT	CGA
CAI0215	CC	AG	TT	CAT	CGT
CAI0216	CC	AG	TT	CAT	CGT

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0217	CC	AG	TA	CAT	CGA
CAI0218	CA	AA	TT	CAT	AAT
CAI0219	CC	AG	TA	CAT	CGA
CAI0220	CC	AA	TT	CAT	
CAI0221	AA	AA	TT	AAT	
CAI0222	CC	AG	TA	CAT	CGA
CAI0223	CC	AG	AA	CAA	CGA
CAI0224	CC	AG	TA	CAT	CGA
CAI0225	AA	AA	TT	AAT	
CAI0227	CC	AA	TT	CAT	
CAI0228	AA	AG	TT	AAT	AGT
CAI0229	AA	AG	TT	AAT	AGT
CAI0230	AA	AA	TT	AAT	
CAI0231	CA	AA	TT	CAT	AAT
CAI0232	CC	AG	TT	CAT	CGT
CAI0233	CC	AG	TA	CAT	CGA
CAI0234	CC	AG	TA	CAT	CGA
CAI0235	CA	AA	TT	CAT	AAT
CAI0236	CC	GG	TA	CGT	CGA
CAI0237	CC	AA	TT	CAT	
CAI0238	CA	AG	TT	CGT	AAT
CAI0239	CA	AA	TT	CAT	AAT
CAI0240	AA	AG	TA	AAT	AGA
CAI0241	CC	AG	TT	CAT	CGT
CAI0242	CC	GG	TA	CGT	CGA

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0243	CA	AA	TT	CAT	AAT
CAI0244	CA	AG	TA	CGA	AAT
CAI0245	CC	AG	TT	CAT	CGT
CAI0246	CC	GG	TT	CGT	
CAI0247	CC	GG	AA	CGA	
CAI0248	AA	AA	TT	AAT	
CAI0249	CC	AG	TA	CAT	CGA
CAI0250	CC	AG	TA	CAT	CGA
CAI0251	CC	AA	TT	CAT	
CAI0252	CA	AA	TT	CAT	AAT
CAI0253	CC	GG	TA	CGT	CGA
CAI0254	CC	GG	TA	CGT	CGA
CAI0255	CC	AG	TA	CAT	CGA
CAI0256	CC	AA	TT	CAT	
CAI0257	CA	GG	TA	CGA	AGT
CAI0259	CC	AA	TT	CAT	
CAI0261	CC	GG	TA	CGT	CGA
CAI0262	CA	AG	TA	CGA	AAT
CAI0264	CA	AG	TA	CGA	AAT
CAI0265	CA	AA	TT	CAT	AAT
CAI0266	CC	AA	TT	CAT	
CAI0267	CA	AG	TA	CGA	AAT
CAI0268	AA	AA	TT	AAT	
CAI0269	CC	AA	TA		
CAI0270	CA	AG	TA		
CAI0271	CC	AG	TA		

PID	-592 (rs1800872)	-1082 (rs1800896)	-3575 (rs1800890)	Haplotype 1	Haplotype 2
CAI0274	AA	AA	TT		
CAI0275	AA	AA	TT		
CAI0276	CA	AG	TA		
CAI0277	CC	GG	TT		
CAI0278	CC	AA	TT		
CAI0279	CA	AG	TA		
CAI0280	AA	AA	TT		
CAI0281	CC	AG	TT		
CAI0282	AA	AA	TT		

Appendix 2: Paper 1

Association of IL-10-Promoter Genetic Variants With the Rate of CD4 T-Cell Loss, IL-10 Plasma Levels, and Breadth of Cytotoxic T-Cell Lymphocyte Response During Chronic HIV-1 Infection

Association of IL-10-Promoter Genetic Variants With the Rate of CD4 T-Cell Loss, IL-10 Plasma Levels, and Breadth of Cytotoxic T-Cell Lymphocyte Response During Chronic HIV-1 Infection

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Background. Interleukin-10 (IL-10) is a potent immunoregulatory cytokine. IL-10-promoter polymorphisms have been shown to affect human immunodeficiency virus type 1 (HIV-1) clinical outcomes but the underlying mechanisms are poorly understood.

Methods. We investigated the relationship between IL-10-promoter variants, plasma cytokine levels, immune responses and markers of disease outcome in antiretroviral-naïve HIV-1 chronically infected individuals from South Africa. Two IL-10-promoter single nucleotide polymorphisms (SNPs) were genotyped in 451 participants. Baseline plasma levels of select cytokines were measured for 112 individuals. Viral load, $CD4^+$ T-cell counts and HIV-1-specific interferon-gamma $CD8^+$ T-cell immune responses were measured at baseline. $CD4^+$ T-cell counts were measured longitudinally and rates of $CD4^+$ T-cell decline computed for 300 study subjects.

Results. The minor IL-10-1082G and -592A variants occurred at frequencies of 0.31 and 0.34, respectively. The -592AA genotype associated significantly with attenuated loss of $CD4^+$ T cells ($P = .0496$). Individuals possessing -1082GG had significantly higher IL-10 levels compared to -1082AA/AG ($P = .0006$). The -592AA genotype was associated with greater breadth of virus-specific $CD8^+$ T-cell responses compared to CC and CA ($P = .002$ and .004 respectively).

Conclusions. IL-10-promoter variants may influence the rate of HIV-1 disease progression by regulating IL-10 levels and the breadth of $CD8^+$ T-cell immune responses.

It is now approximately 3 decades since the human immunodeficiency virus type 1 (HIV-1) was first described, and the virus has since spread to become a pandemic with high morbidity and mortality. Almost two-thirds of the world's HIV-infected individuals are found in sub–Saharan Africa, including South Africa [1]. Although antiretroviral drugs are now widely available for the clinical management of HIV-1 infection, significant challenges in the roll-out and subsequent lifelong use of these drugs remain, and it is unlikely that the spread of HIV-1 will be substantially curtailed without a preventive vaccine or immunotherapy. However, vaccine development efforts have been significantly hampered by the limited knowledge of the biological factors that underlie HIV-1 pathogenesis, and so far, only modest success or failure has been achieved with candidate HIV-1 vaccines that have undergone large-scale human trials [2–4]. The considerable variation in the natural history of HIV/AIDS and disproportionate global distribution of HIV infection present an opportunity to study

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biological factors that may influence HIV/AIDS pathogenesis, and have the potential to be manipulated for effective vaccination or therapy [5, 6].

Geographical differences of host genetic factors that influence HIV/AIDS exposure or infection are well illustrated in the distribution of the CCR5 chemokine receptor mutation CCR5-Δ32, which has been associated with protection against HIV-1 infection by R5 HIV-1 variants [7, 8]. Studies have shown that the frequency of the CCR5-Δ32 alleles is higher among Northern Europeans, decreasing geographically further south [9]. Likewise, the HLA locus, which codes for HLA class I molecules (which function to present pathogen-derived peptides on the cell surface of infected cells for recognition by $CD8^+$ T lymphocytes [5, 9]), displays significant natural variation among geographically diverse populations, and has been associated with differences in HIV/AIDS disease progression and natural viral control [9–11].

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine, which plays a key role in regulating the immune response [12, 13]. IL-10 has also been shown to downregulate the expression of proinflammatory cytokines as well as the expression of major histocompatibility complex class I and II molecules [14–17]. Previous studies have focused on the 3 classic single-nucleotide polymorphisms (SNPs) found in the proximal promoter region [18–21]. These polymorphisms are found at positions -1082 (rs1800896), an A to G transition; -819 (rs1800871), a C to T transition; and -592 (rs1800872), a C to A transversion. The 819 and -592 mutations are in complete linkage disequilibrium. IL-10 variants are associated with differential IL-10 production [19, 22–24]; -1082G with high IL-10 production and the -592A with low IL-10 production. Genetic association data suggests that IL-10 variant -592A, a low IL-10 producer variant, is linked with increased susceptibility to HIV-1 infection and an accelerated progression to AIDS, particularly in the late stages of the disease among European Americans [20, 21, 25]. Consistent with these data, it was demonstrated in an African cohort that survival was doubled in carriers of the IL-10-1082G allele, which is associated with increased IL-10 production [18]. Therefore, there is evidence that suggests that IL-10 polymorphisms associated with increased IL-10 production have a protective role against disease progression, possibly by decreasing the chronic immune activation that is a major factor in HIV pathogenesis.

On the other hand, we have recently shown in a cohort of high-risk black African women that although the IL-10 polymorphisms -1082AA and -592AA associated with decreased IL-10 production were overrepresented among seroconverters compared with those who remained HIV-1 negative in longitudinal follow-up, these polymorphisms also associated with high viral loads and low CD4 counts during the acute/early phases of infection, suggesting that the effects of IL-10 polymorphisms may be infection-phase dependent and that high IL-10 levels during the early phase may be detrimental [26]. Mechanistic studies of

Table 1. Baseline Characteristics of the Study Group Based on Genotype

Characteristic	-1082 Genotype			-592 Genotype			Total (n = 300)
	AA (n = 141)	AG (n = 122)	GG (n = 35)	CC (n = 142)	CA (n = 127)	AA (n = 30)	
Age, median years (IQR)	32 (27–37)	31 (27–38)	30 (26–35)	30 (26–36)	31 (28–37)	34 (29–37)	31 (27–37)
Sex, female (%)	109 (77)	102 (84)	32 (91)	116 (82)	104 (82)	24 (80)	245 (82)
Baseline pVL >100 000 copies/mL (%)	52 (37)	46 (38)	9 (26)	48 (34)	47 (37)	12 (40)	107 (36)
Baseline CD4 >350 cells/μL (%)	84 (60)	76 (62)	25 (71)	92 (65)	76 (60)	17 (57)	186 (62)
Median follow-up, months (IQR)	24.2 (14.4–37.8)	24.4 (12.9–41.0)	28.9 (21.9–45.3)	24.7 (14.5–41.0)	24.2 (15.4–41.5)	27.1 (15.9–42.6)	24.7 (15.0–41.0)

Abbreviations: IQR, interquartile range; pVL, plasma viral load.

Table 2. Baseline Viral Load and CD4$^+$ T-Cell Count Based on IL-10 Genotype

	−1082 Genotype				−592 Genotype			
	AA	AG	GG	*P* value	CC	CA	AA	*P* value
Baseline CD4 (cells/μL)	341	375	431	0.23	397	339	341	.22
Log mean pVL (log copies/mL)	4.8	4.8	4.7	0.40	4.8	4.8	4.8	.41

Abbreviation: pVL, plasma viral load.

the lymphocytic choriomeningitis virus (LCMV) in mice showed that IL-10 gene knock-out or signaling blockade enhanced T-cell immune responses, resulting in rapid viral elimination and the development of antiviral memory T-cell responses [27, 28]. We have also shown in vitro that IL-10 blockade in peripheral blood mononuclear cells (PBMCs) from HIV-infected individuals resulted in restoration of proliferative and effector CD4 T-cell function [29]. IL-10 is also reported to enhance detrimental deletion of dendritic cells by natural killer cells, further exacerbating immune dysfunction in chronic HIV-1 infection [30]. Taken together, these studies suggest a complicated but significant role for IL-10 in viral pathogenesis, and considering that manipulation of the IL-10 pathway to boost antiviral immune responses and improve vaccine effectiveness has been suggested, there is a clear and urgent need to better decipher underlying mechanisms of pathogenesis for this immunoregulatory cytokine, particularly in geographical regions most severely affected by the HIV-1 epidemic, as this may have implications for immunotherapeutic strategies and vaccine design.

Therefore, the purpose of this study was to investigate the frequency of IL-10-promoter polymorphisms in a large cohort of antiretroviral-naive chronically HIV-1-infected predominantly Zulu/Xhosa individuals to determine whether these polymorphisms affect markers of HIV-1 disease progression; namely, viral load, CD4$^+$ T-cell counts, and the rate of CD4$^+$ T-cell decline. We also wanted to determine whether these polymorphisms are associated with differential levels of select pro- and anti-inflammatory plasma cytokines. Furthermore, we sought to explore the link between these polymorphisms and levels of cytotoxic T-cell immune responses, which have been shown to play a role in the control of HIV-1 replication. Our data suggest that IL-10-promoter polymorphisms may modulate HIV-1 pathogenesis, possibly through effects on plasma IL-10 levels and the breadth of immune responses, among other mechanisms.

MATERIALS AND METHODS

Study Population, Materials, and Methods

The HIV Pathogenesis Programme (HPP) Sinikithemba cohort is described in detail elsewhere [31]. This cohort is based at McCord Hospital in Durban, South Africa. The cohort was established in August 2003, with 451 antiretroviral-naive chronically HIV-1-infected adult study subjects enrolled until June 2006.

CD4 cell counts and plasma viral loads were performed routinely for study participants. CD4$^+$ T-cell counts were enumerated by flow cytometry (Becton Dickinson, San Jose, CA), while plasma viral loads were measured using the Roche Amplicor version 1.5 assay (Roche, NJ). CD4$^+$ T-cell counts were performed at 3-month and plasma viral loads at 6-month intervals. The number and magnitude of HIV peptides targeted by cytotoxic T lymphocytes (CTLs) were measured at baseline by interferon-γ (IFN- γ) enzyme-linked immunospot (ELISPOT) assay using a panel of 410 overlapping peptides spanning the HIV-1C proteome [31, 32].

IL-10 genotype data for SNPs -1082 and -592 were generated using predesigned TaqMan SNP Genotyping assays (Applied Biosystems, CA). Plasma IFN-γ, IL-2, IL-6, IL-10, and tumor necrosis factor (TNF)–α concentrations were determined by Luminex, using the Millipore Milliplex MAP High Sensitivity Human Cytokine Kit.

Different methods of statistical analysis were used to determine the association and correlation of IL-10 variants/levels with aforementioned biomarkers. We used the χ^2 test to compare the allelic frequencies of the IL-10 variants, confirming their fit to Hardy–Weinberg equilibrium. Kruskal–Wallis tests were used to determine the association between IL-10 variants and viral load or CD4$^+$ T-cell count. The CD4 decline over 24 months of follow-up was estimated using multivariate mixed effects models. The Kruskal–Wallis test was used to determine the association between IL-10 variants and magnitude or breadth of immune responses. To determine if there was an association between IL-10 variant and plasma IL-10 concentration, the Kruskal–Wallis test was used. The Pearson product moment correlation test was used to determine the association between IL-10 concentration and markers of HIV-1 pathogenesis or plasma cytokines.

RESULTS

IL-10-Promoter Genotyping

The 2 IL-10 promoter positions -1082 and -592 were polymorphic in this cohort of HIV-1C chronically infected individuals. The allele frequencies of the minor -1082G and -592A polymorphisms

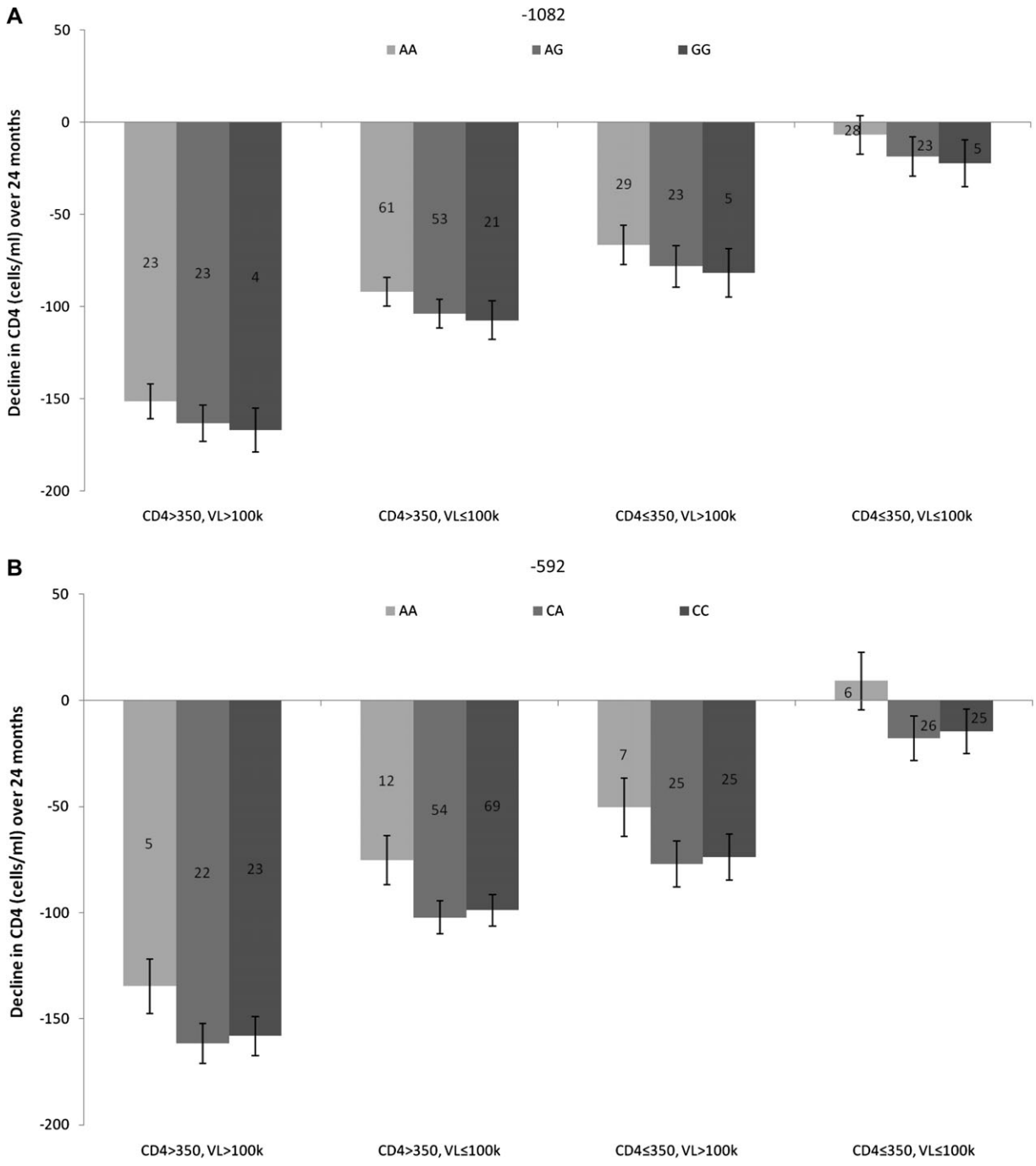

Figure 1. The rate of $CD4^+$ T-cell decline over 24 months based on IL-10 variants. Data was stratified into different groups based on viral load and CD4 count (ie, CD4 >350, VL >100 000; CD4 >350, VL ≤100 000; CD4 ≤350, VL >100 000; and CD4 ≤350, VL ≤100 000). The numbers in the bars indicate the number of people in each category. *A*, The rate of $CD4^+$ T-cell decline over 24 months based on -1082 genotype. The low IL-10-producing -1082AA groups tended to have an attenuated loss of CD4 cells as compared to the other groups; however, this was not significant ($P = .15$). *B,* The rate of $CD4^+$ T-cell decline over 24 months based on -592 genotype. The low IL-10-producing -592AA group had an attenuated loss of CD4 cells over 24 months. The was a significant association between -592 genotype and CD4 cell loss ($P = .0496$). Abbreviations: IL, interleukin; VL, viral load; k, thousands.

were 0.31 and 0.34, respectively. Allele frequencies were confirmed by χ^2 tests to be in Hardy–Weinberg equilibrium for the entire cohort and for subgroups analyzed in this study. The baseline characteristics of the cohort are shown in Table 1. The median age of the study group was 31 years, 82% were female, 36% had a baseline viral load (VL) >100 000 copies/mL, 62% had

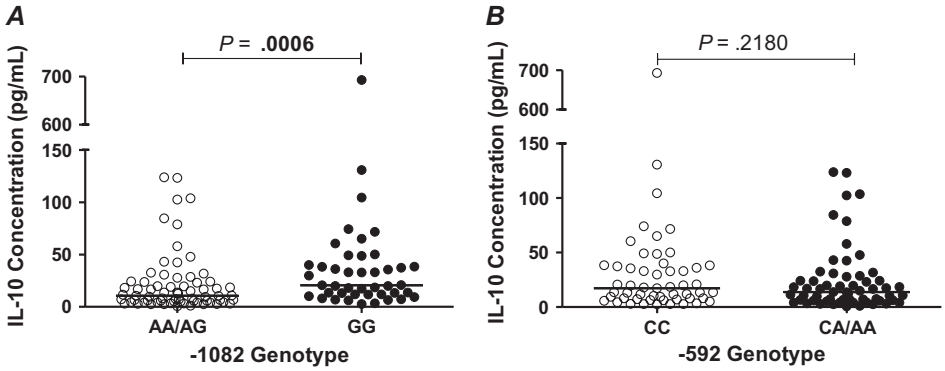

Figure 2. The association of IL-10 expression based on IL-10 genotype. *A*, the association of IL-10 expression based on -1082 genotype. Genotypic groups were grouped according to a recessive model of inheritance. We found a significant association between -1082 genotype and IL-10 expression ($P = .0006$). *B*, The association of IL-10 expression based on -592 genotype. Genotypic groups were grouped according to a dominant model of dominance. There was no significant association between -592 genotype and IL-10 expression ($P = .2180$). Abbreviations: IL, interleukin; VL, viral load; k, thousands.

a baseline CD4 count >350 cells/μL, and had a median follow-up of 24.7 months.

Viral Load, $CD4^+$ T-Cell Count, and CD4 Decline

For the 409 individuals with baseline data, we investigated the association between the IL-10 genotypes and baseline viral load and $CD4^+$ T-cell counts. Table 2 shows the association between baseline viral load and $CD4^+$ T cells. In this cross-sectional analysis, we found no association between any IL-10 variant and viral load or $CD4^+$ T-cell count.

We investigated the rate of $CD4^+$ T-cell decline in 300 individuals with follow-up data. Figure 1 shows the rate of $CD4^+$ T-cell decline over 24 months based on genotype. CD4 decline was stratified according to CD4 and viral load (ie, CD4 >350, VL >100 000; CD4 >350, VL ≤100 000; CD4 ≤350, VL >100 000; CD4 ≤350, VL ≤100 000). The low-IL-10-producing -1082AA genotype had an attenuated CD4 cell loss compared with the -1082AG or -1082GG groups (Figure 1*A*). This trend was seen in each strata; however, there was no significant association ($P = .15$). The low-IL-10-producing -592AA genotype had an attenuated loss of CD4 cells over all strata (Figure 1*B*), with a borderline significant association between the -592 genotype and CD4 decline over 24 months ($P = .0496$). Overall, low-IL-10-producing variants had an attenuated loss of CD4 cells over all strata in this study cohort.

Cytokine Expression Analysis

We next investigated the association between IL-10-promoter polymorphisms and plasma IL-10 and/or plasma proinflammatory cytokines IFN-γ, IL-2, IL-6, and TNF-α in a subset of 112 individuals. To determine if IL-10 levels associate with IL-10 polymorphisms in an African setting of chronic HIV-1C infection, we investigated if plasma IL-10 levels associate with IL-10 variants (Figure 2). We grouped the genotypes according to dominance patterns previously described by Shin et al. [21]. Figure 2*A* shows that based on a recessive model, the -1082GG group had significantly higher median plasma IL-10 concentration compared with the combined -1082AA/AG groups ($P = .0006$). Figure 2*B* shows that when considering a dominant model [33], the -592CC group had a higher median IL-10 concentration as compared with the combined -592CA/AA groups; however, this was not significant ($P = .2180$). In a cross-sectional analysis, and in contrast to what had been seen in other cohorts of different ethnicities located in other geographic areas [29], IL-10 plasma levels did not significantly correlate with viral load (Pearson correlation = 0.08465, $P = .3838$), CD4 count (Pearson correlation = −0.02797, $P = .7749$), the breadth of immune responses (Pearson correlation = −0.0613, $P = .5304$), or the magnitude of immune responses (Pearson correlation = 0.0597, $P = .5412$), data not shown. As IL-10 is a major inhibitory immunoregulator, we next examined whether the different IL-10-promoter polymorphisms were associated with distinct cytokine profiles in peripheral blood. Figure 3 represents the analysis of the 5 cytokines that were measured. Figure 3*A* shows that there was significant positive correlation between the levels of each of the proinflammatory cytokines (IFN-γ, IL-2, and IL-6 and TNF-α) and IL-10 levels ($P < .0001$, Spearman rank correlation [ρ]). Figure 3*B* and 3*C* shows that, overall, IL-10 dominated the measured plasma cytokine levels in this chronic HIV-1C setting irrespective of the IL-10 genotype.

Breadth and Magnitude of Immune Responses

In the LCMV murine model of chronic viral infection, IL-10-deficient mice showed an increased frequency of tetramer-positive virus-specific $CD8^+$ T cells, and IL-10 receptor blockade

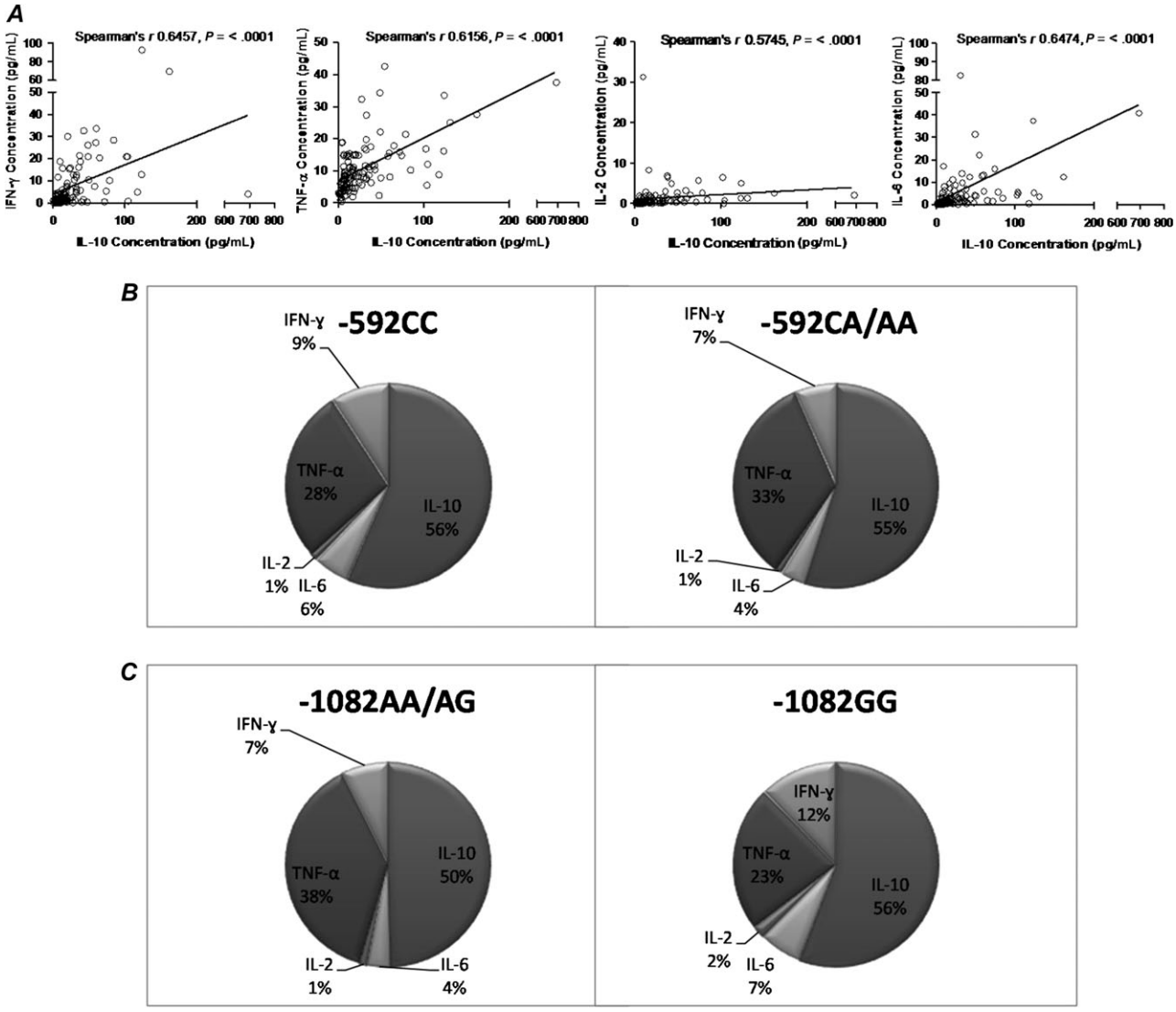

Figure 3. Cytokine expression during chronic HIV-1C infection. IL-10, IFN-γ, TNF-α, IL-2, and IL-6 were measured by Luminex methodology in 112 individuals. *A*, The correlation between IL-10 and IFN-γ, TNF-α, IL-2, and IL-6. Overall, all the cytokines had a significantly positive correlation with IL-10 expression ($P < .0001$ for each cytokine, Spearman ρ). *B*, Proportion of cytokine expression based on IL-10 -592 genotype. IL-10 seemed to dominate cytokine expression overall. *C*, Proportion of cytokine expression based on IL-10 -1082 genotype. IL-10 seemed to dominate cytokine expression overall. Abbreviations: HIV, human immunodeficiency virus; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

increased IFN-γ production by virus-specific $CD8^+$ T cells [27, 28] We therefore reasoned that IL-10 variants that affect IL-10 production and influence disease progression may also be linked with the magnitude (number of IFN-γ-producing cells per million PBMCs) and breadth (number of HIV peptides targeted by CTLs) of HIV-1-specific immune response in vivo, as measured by IFN-γ ELISPOT. We thus investigated the association between IL-10 variants and the magnitude and breadth of $CD8^+$ T-cell immune responses. Figure 4*A* and 4*B* shows the association between the magnitude of immune responses based on the 1082 and -592 genotypes, respectively. We found no significant association between the magnitude of HIV-1-specific immune responses and -1082 genotype ($P = .44$) or the -592 genotype ($P = .17$). We then assessed the breadth of immune responses in relation to IL-10 genotype. We found no significant association between -1082 genotype and the number of HIV peptides targeted by CTLs ($P = .2316$). However, there was a significant association between the number of HIV peptides targeted and the -592 genotype ($P = .0069$). The low-IL-10-producing -592AA group had a median of 12 HIV peptides versus 7 peptides targeted for the -592CC or -592CA genotypes ($P = .002$ and 0.004, respectively).

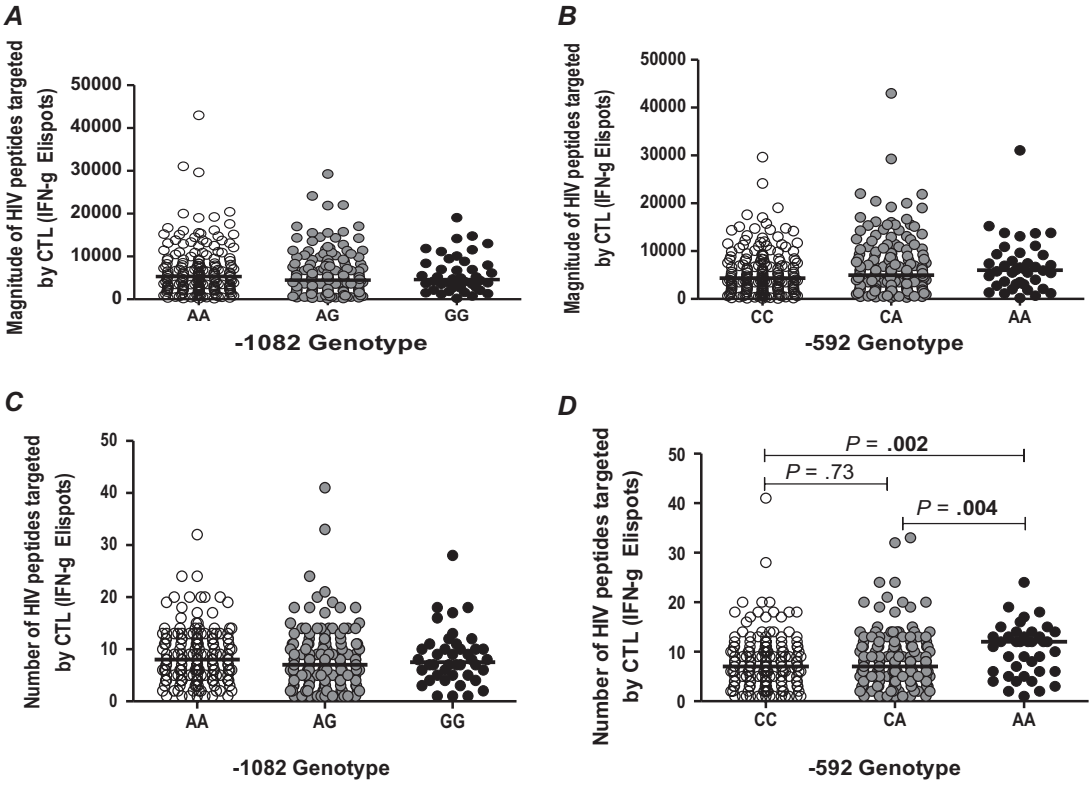

Figure 4. Magnitude and breadth of immune responses based on genotype. *A*, the magnitude of HIV peptides targeted by CTLs (IFN-γ) based on -1082 genotype. There was no significant association between -1082 genotype and the magnitude of immune responses ($P = .044$). *B*, The magnitude of HIV peptides targeted by CTLs (IFN-γ) based on -592 genotype. There was no significant association between -592 genotype and the magnitude of immune responses ($P = .17$). *C*, The number of HIV peptides targeted by CTLs (IFN-γ) based on -1082 genotype. There was no significant association between the number of HIV peptides targeted by CTLs and -1082 genotype ($P = .23$). *D*, the number of HIV peptides targeted by CTLs (IFN-γ) based on -592 genotype. There was a significant association between the number of HIV peptides targeted by CTLs and -592 genotype ($P = .007$). The -592AA group targeted a significantly larger number of HIV peptides as compared to the -592CC or 592CA groups ($P = .002$ and .004, respectively). Abbreviations: HIV, human immunodeficiency virus; CTLs, cytotoxic T lymphocytes; IFN, interferon.

DISCUSSION

In this study, we sought to extend earlier observations on the modulation of HIV-1 infection by genetic polymorphisms of the IL-10 promoter to an investigation of possible underlying mechanisms in vivo. We therefore characterized IL-10-promoter SNPs in a large, predominantly black African cohort chronically infected with HIV-1 subtype C. IL-10 polymorphisms were investigated for association with biomarkers of disease progression; namely, plasma viral load, $CD4^+$ T-cell counts, and the rate of $CD4^+$ T-cell decline. These polymorphisms were then analyzed for association with plasma IL-10 and select proinflammatory cytokines levels (as this had not previously been investigated in an HIV setting), and the breadth and magnitude of $CD8^+$ T-cell immune responses. Our data suggest an association between IL-10-promoter genotypes with the rate of $CD4^+$ T-cell loss during chronic HIV-1 infection, an association with plasma IL-10 levels, a predominance of the anti-inflammatory IL-10 over proinflammatory cytokines in the plasma of HIV-1-infected individuals, and an effect of IL-10 polymorphisms on the breadth but not the magnitude of $CD8^+$ T-cell immune response.

Our data suggest that in an African setting of chronic HIV-1 infection, IL-10 variants may influence the rate of disease progression. Our investigation into the role of IL-10 in HIV-1C pathogenesis showed that these IL-10-promoter polymorphisms that have been previously shown to be associated with differing levels of IL-10 expression [19, 22–24] significantly associate with differential IL-10 expression in an HIV setting. Low-IL-10-producing -592 variants showed a trend toward attenuated $CD4^+$ T-cell loss. These data differ from earlier studies in European and African cohorts in which high-IL-10-producing genotypic variants were associated with attenuated $CD4^+$ T-cell loss or progression to AIDS [18, 21]. A possible explanation for these differences might be that the effect of IL-10-promoter variants on HIV-1 pathogenesis is infection-phase dependent, as we have previously suggested [26]. This interpretation is also consistent with the observations of Shin and colleagues [21] who found that high-IL-10-producing genetic variants were

protective against disease progression, particularly during late states of infection, and yet the genetic removal of or blockade of IL-10 in a mouse model of chronic viral infection has been shown to result in viral clearance [27, 28]. Our hypothesis is that high IL-10 production (and by extension high-IL-10-producing promoter genotypes) are detrimental during early HIV-1 infection because of downregulation of antiviral adaptive and innate effector mechanisms [30, 34, 35] but beneficial in late stages of infection because of anti-inflammatory effects of IL-10 and direct-inhibition viral replication in macrophages [36–38]. It is also possible that different strains of HIV-1 induce different levels of IL-10, as has been recently demonstrated for HIV-1 B versus C trans-activating proteins [39], although this explanation is unlikely to account for the different results observed in our study versus the other African study [18] since both were done in settings where HIV-1 subtype C predominates. Alternatively, IL-10 SNPs may have underlying undetermined epigenetic or environment modulatory factors, or these SNPs may be in linkage disequilibrium with other genes with a modulatory role on attenuated $CD4^+$ T-cell loss. Further studies will be needed to discriminate between these possibilities.

Overall, IFN-γ, IL-2, IL-6, and TNF-α had a significant positive correlation with IL-10 expression. Showing that in an HIV-1C setting, both pro- and anti-inflammatory cytokines are upregulated. However, we found that the proportion of IL-10 expression seemed to dominate over the expression of the other cytokines. The dominance of IL-10 expression suggests that as the production of proinflammatory cytokines increases, the production of IL-10 also increases to reduce inflammation and activation.

The -592A variant, associated with low IL-10 production, significantly associated with a higher number of HIV-1 peptides targeted by CTLs. These data are consistent with results from mechanistic studies on the LCMV mouse model, which show that the removal or blockade of IL-10 enhance T-cell immune responses [27, 28]. Similarly, results from our study of IL-10 blockade in vitro resulted in restoration of proliferative and effector CD4 T-cell function [29]. Lower IL-10 levels may allow for the expression of HLA class I and II molecules, which in turn increases the presentation of pathogen-derived peptides on the cell surface of infected cells, for recognition by $CD8^+$ T lymphocytes. However, as IL-10 levels did not correlate with plasma viral load, $CD4^+$ T-cell count, or the breadth of immune responses, IL-10 polymorphisms may contribute to the quality of immune responses via a complex pathway that has yet to be elucidated. Alternatively, differential levels of IL-10 secretion that are critical for paracrine cell–cell interactions may not be reflected by differences in IL-10 plasma levels.

There are a number of limitations to this cohort study. First, the time since HIV-1 infection is unknown for study participants, which may have introduced a survivor bias in analysis of IL-10 polymorphisms since these have been shown to affect survival [18]. Second, since time of infection was unknown, we may be analyzing individuals at different phases of infection together, although we stratified our data according to viral loads and CD4 cell counts to partially mitigate for this limitation. This study also focused on 2 proximal IL-10-promoter polymorphisms, only a subset of IL-10 SNPs shown to affect IL-10 production. Finally, IL-10 and other cytokines can be induced by a plethora of pathogens, and we lacked data on coinfection status of participants in this study.

In conclusion, our study highlights the complex role that IL-10 and IL-10 genetic variants may play in HIV-1 pathogenesis. We show that IL-10-promoter polymorphisms may play a role in the rate of CD4 T-cell loss in chronic HIV infection, and affect IL-10 plasma levels and the breadth of anti-HIV $CD8^+$ T-cell immune responses. However, to fully understand the effects and underlying mechanisms of IL-10 in HIV-1C pathogenesis, expanded analysis is required of the proximal and distal SNPs. Additional mechanistic studies will also be required in order to fully understand how best to target the IL-10 pathway for effective immunotherapy or a vaccine.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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